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13. ABSTRACT *(Maximum 200 Words)*

It is proposed that reversing the effects of CD59, a tumor cell expressed complement inhibitor, will allow effective immune-mediated clearance of tumor cells and improve prospects for successful immunotherapy. This study investigates the role of complement and CD59 in tumorigenesis. CD59 function is species selective, and we have determined that the expression of either rat or mouse CD59 on breast human breast cancer cells protects them from lysis by rat and mouse complement, respectively. We have determined species selectivity of human, rat and CD59, an important consideration for establishing human models of human cancer in rodents for the study of complement.We have further Identified the individual residues that confer human CD59 species selective activity. This data is an important step toward identifying the three dimensional structure of the CD59-C9 peptide ligand complex and may assist in design of CD59 inhibitors. Finally, we have established for the first time that CD59 expressed on the surface of a tumor cell can significantly promote tumor growth

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S Tomlinson

PI - Signature Date

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INTRODUCTION

Complement effector systems involved in the immune response to tumor cells include amplification of inflammatory response, recruitment of immune effector cells and direct and NK cell mediated cytolysis. It is hypothesized that complement regulatory proteins expressed on the tumor cell surface promote tumorigenesis and present a barrier to effective complement-mediated immunotherapy. We propose that reversing the effects of tumor-expressed complement inhibitors will allow effective immune-mediated clearance of tumor cells and improve prospects for successful immunotherapy.

Membrane inhibitors of complement protect tumor cells from cytolytic complement attack *in vitro*. CD59 and usually DAF and/or MCP are expressed by virtually all breast and other primary tumors and tumor cell lines that have been examined (1-4). CD59 neutralization *in vitro* by anti-CD59 mAbs enhance complement-mediated lysis of breast tumor cells (1). Thus, effective lysis of breast tumor cells by complement *in vitro* requires that their resistance to complement be overcome, an important consideration for complement-dependent immunotherapy using mAbs. The current project is focussed on the study of the complement inhibitory protein, CD59.

BODY

TASK 1: Months 0-6: IN VITRO EXPERIMENTS: Confirmation of the role of CD59 in conferring protection against antibody-targeted complement lysis of tumor cells. Will transfect human tumor cell lines with rat CD59 and select expressing populations. Will determine if transfected cells have increased resistance to rat complement.

This task has been completed (and extended to include mouse CD59). The data is published (5) and the paper is included in the appendix. The data is summarized below.

Summary

Breast cancer cell line MCF7 cells were transfected with rat or mouse CD59 cDNA, and cell populations stably expressing high levels of recombinant rodent CD59 were isolated by cell sorting. Transfected cell populations were tested for their susceptibility to complement-mediated lysis to determine whether expression of rodent CD59 correlated with increased resistance to rodent complement. Untransfected MCF7 cells were relatively resistant to lysis by homologous human complement, but were effectively lysed by both rat and mouse complement. The expression of either rat or mouse CD59 on MCF7 cells however, protected them from lysis by rat and mouse complement, respectively. MCF7 cells expressing rat CD59 were almost totally resistant to lysis by rat complement. The increased rat complement resistance of rat CD59 transfected MCF7 cells was reversed by the addition of anti-rat CD59 blocking mAb 6D1, thus confirming that the heterologously expressed rodent CD59 is responsible for providing the observed protection from rodent complement-mediated lysis. Data further show the relative activities of each CD59 protein against heterologous sera.

Discussion

The demonstration that heterologous (nonhuman) cells transfected with human CD59 display increased resistance to lysis by human complement provides direct and unequivocal evidence that human CD59 inhibits human complement-mediated cell lysis. The phenomenon of species selective activity allowed us to use a reciprocal approach to determine directly the functional significance of CD59 expressed on human breast tumor cells. Data generated is relevant to establishing rodent models for the study of complement and complement inhibitors in tumor growth and control.

TASK 2: Months 0-12: IN VITRO EXPERIMENTS: Determination of the effect or rat complement on human breast cancer cells. First, different breast tumor cell lines will be screened for CD59 expression. Sensitivity of CD59 positive cells to rat serum will be assayed. Cells will be sensitized

to complement using tumor cell specific antibodies. Will repeat experiments using purified complement components to show if CD59 is inhibiting rat complement protein C9.

This task has been performed using the MCF7 cell line and results published (5). Paper is included in appendix.

Studies using other breast cancer cell lines have been performed, namely BT474, T47D and SKBr3. All cell lines expressed high levels of CD59 and also inhibitors of complement activation, DAF and MCP. The data was similar to that obtained for MCF7 (5). All cell lines were relatively susceptible to lysis by rat complement (but not human complement), making them potential candidates for proposed in vivo studies.

TASK 3: Months 6-18: IN VITRO EXPERIMENTS: Targeting CD59 inhibitory antibodies to breast tumor cells. Will confirm that rat adenocarcinoma 13762 cells express CD59. Then confirm their susceptibility to rat complement after neutralization of rat CD59. If successful, will isolate 13762-specific antibodies and attempt to target anti-rat CD59 mAbs to 13762 cell surface by means of 13762-specific antibodies and biotin-avidin bridges.

We have determined that rat adenocarcinoma 13762 cells express CD59, and also Crry, a complement inhibitor of activation (fig. 1).

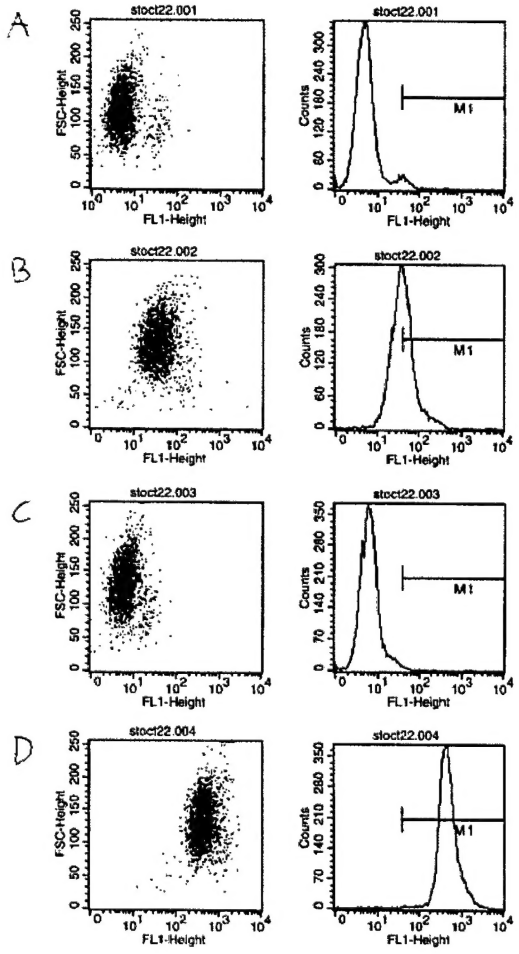

Figure 1. Endogenous expression of CD59 and Crry by rat adenocarcinoma 13762 cells. Cells were stained by immunofluorescence using monoclonal antibodies to rat CD59 (B) or rat Crry (D) as primary antibodies. Isotype matched antibodies of irrelevant specificity were used for controls (A and C).

We have determined that blocking CD59 expressed on 13762 cells enhances their susceptibility to rat complement (Fig 2), but that the 13762 cells remained fairly resistant. Rat 13672 cells were found to express

high levels of Crry (fig.1), an inhibitor of complement activation, which may account for the only modest enhancement of complement-mediated lysis when CD59 function is blocked. Crry blocking studies are underway.

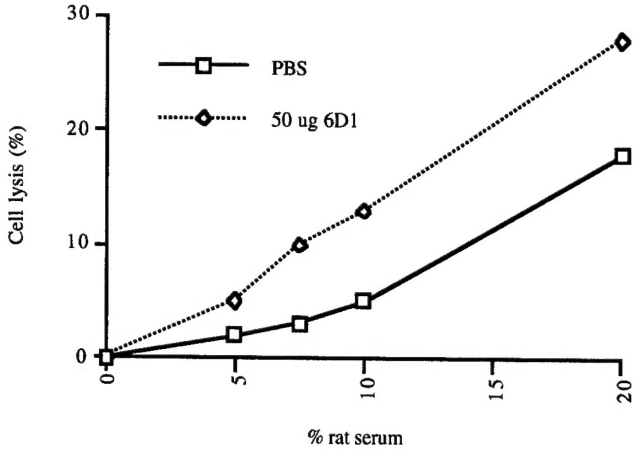

Figure 2. Rat complement-mediated lysis of 13762 cells in presence and absence of an antibody (6D1) that blocks CD59 function. Cells were preincubated with 50 ug/ml 6D1 (30 min/4oC), washed and incubated with senstizing antibody, and the indicated concentration of rat serum added. Lysis was then determined after 1 h/37oC. Higher concentrations of 6D1 antibody did not enhance lysis any further.

Methods

Rat CD59 and rat Crry expression on 13762 cells was determined by flow cytometry by standard procedures (6). Blockade of CD59 with anti-CD59 antibody and complement lysis assays were performed as described (5) (paper in appendix).

Discussion

Blocking CD59 function did enhance complement-mediated lysis of 13762 cells, but not as significantly as has been reported for other human cancer cell lines. This data questions the relevance of trying to isolate 13762-specific antibodies and attempting to target anti-rat CD59 mAbs to 13762 cell surface by means of 13762-specific antibodies and biotin-avidin bridges. We found that 13762 cells express high levels of another complement inhibitor, Crry, and we will attempt blocking studies with antibodies to Crry to determine whether Crry may make a better target than CD59 for the proposed complement inhibitor blocking studies. We will also perform complement lysis/CD59 blocking experiments using other rat tumor cell lines in an attempt to find a cell line that behaves similarly to many human cell lines.

TASK 4: Months 6-24: IN VITRO: Will test rat and human breast tumor cell lines transfected with HER2 and rat CD59, respectively, for sensitivity to rat complement. Will assess ability of tumor specific antigens to target transfected cells. Will attempt to target anti-rat CD59 antibodies to transfected cell surface by means of tumor specific antibody and biotin-avidin bridge.

Results

This task is related to tasks 1 and 2, and data presented above is also relevant. Two breast cancer cell lines have been transfected with rat CD59: MCF7 (5) (appendix) and BT474 (not shown). Their susceptibility to rat complement has been determined (task 2). Cell lysis of MCF7 and rat CD59 transfected MCF7 is reported (5). The lysis of untransfected and transfected BT474 by rat complement (using an anti-BT474 membrane complement-sensitizing antiserum) was the same as that for MCF7 and is not shown.

BT474 is a HER2 positive cell line. MCF7 is a MUC1 positive cell line. We have shown that certain antibodies against the breast tumor-specific antigens HER2 and MUC1 are able to target breast cancer cell lines and activate complement (fig. 3). The anti-HER2 mAb used was from a commercial source. We have obtained an anti-HER2 IgG2b hybridoma, and will test this antibody (since commercial source is not

available in high quantity for in vivo studies). The hybridoma could not be grown in culture, and we are currently adapting the cell line for in vitro growth.

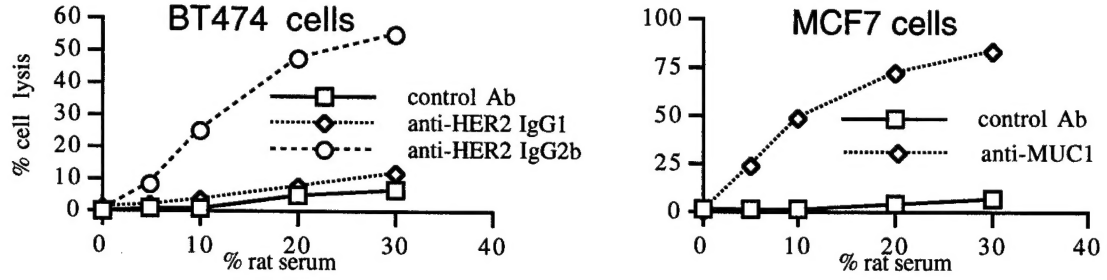

Fig 3. Breast tumor cell lines can be sensitized to heterologous complement by antibody recognizing tumor-specific (overexpressed) antigen. Standard assay procedures were followed (see above). The anti-MUC1 antibody source used was rabbit polyclonal antiserum. The anti-HER2 antibodies were purified mAbs used at 20 ug/ml.

An IgM monoclonal antibody directed against the breast cancer-associated antigen MUC1 (BC3,(7)) also sensitized MCF7 cells to lysis by rat complement, but was less effective than the polyclonal antiserum shown above (data not shown). We have also obtained a complement activating IgG3 anti-MUC1 antibody. We have been unable to produce good complement-mediated lysis of MCF7 with the monoclonal antibodies. However, we have discovered a problem with the downregulation of MUC1 on multiply passaged MCF7 cells and are taking steps to correct this by growing MCF7 cells on agar. We will re-evalaute the mAbs following upregulation of MUC1 on MCF7 cells to determine if they represent effective complement-activating antibodies (MCF7 express high levels of MUC1 when grown in vivo).

Of relevance to antibody targeting and engineering of antibody-targeted constructs, we have produced and characterized IgG-CD59 targeted fusion proteins. Results are published (8) and paper is included in appendix.

Discussion

The combined presented data (tasks 1-3) indicate that endogenous CD59 expressed on human tumor cells implanted into rodents is unlikely to provide effective protection against complement attack when tumors are targeted by complement activating antibodies. The relative ineffectiveness of human CD59 against rat and mouse complement presents a serious hindrance for studies aimed at determining the protective role of CD59 (and other complement inhibitors) in rodent hosts bearing human cancers. The current data establishes the feasibility of using human cancer cells expressing rodent CD59 to show, in vivo, the regulatory effects of CD59 on complement-mediated tumor cell lysis. Current efforts are directed at identifying a suitable complement-activating monoclonal antibody for in vivo studies.

TASK 5: Months 0-36: Will use molecular modelling techniques to determine C9 peptide ligand for CD59 binding, and determine three dimensional structure of the CD59-C9 peptide ligand complex.

We have identified the individual residues that confer human CD59 species selective activity. The data has been published (9) and the paper is included in the appendix. Defining the functional site of CD59 may assist in design of CD59 inhibitors on tumor cells. This data is an important step toward identifying the three dimensional structure of the CD59-C9 peptide ligand complex.

TASK 6: Months 6-36: IN VIVO: Continuation of task 1. Determine which human breast cancer cell lines grow in nude rats (about 20 rats required). Will Use cell line that developed tumors and that has been successfully transfected with rat CD59 to seed nude rats. Will then determine the effect of tumor-specific antibodies on growth of these cells in rats (about 40 rats required).

Growth of breast cancer cell lines

We have established conditions for estrogen-supplemented growth of MCF7 and BT474 cell lines in Rowett nude rats. Between 5 x 10^5 and 1 x 10^7 cells injected per site with matrigel produce tumors. The cell line SKBR3 did not grow in nude rats under the conditions tested (up to 1 x 10^7 cells injected per site with and without matrigel).

Growth of breast cancer cell lines transfected with rat CD59

MCF-7 cells stably expressing rat CD59 and control transfecetd MCF-7 cells were innoculated into nude rats to determine whether the expression of rat CD59 provided a growth advantatage. There was no significant difference in either the appearance of tumor or tumor growth rate between the test and control groups. We also analyzed C3 deposition on the tumor cells, and found no significant C3 deposition. Thus, it is possible that no difference was observed because cmplement was not activtated on the MCF-7 cell membrane (see results for in vivo growth of neuroblastoma transfected with rat CD59 below). These experiments will now be repeated with MCF7 and transfected MCF7, but in the presence of an anti-tumor Ab that can activate complement. We also plan to extend these studies using Crry transfected human breast cancer cell lines.

Growth of a neuroblastoma cell line transfected with rat CD59

We found that a neuroblastoma cell line, LAN-1, spontaneously activated rat complement. We also have an abundant source of an effective complement activating antibody against a surface antigen of LAN-1 (GD2 antigen). The hypotheses that we are trying to establish relating to the role of complement inhibitors in antibody-mediated immunotherapy are likely to be relevant for various tumor types, therefore we also performed experiments with this cell line which has suitable characteristics for a rat model, and for which good reagents are already available. The data shown in fig. 3 show that LAN-1 transfected with rat CD59 have a significant growth advantage over LAN-1 transfected with a control protein (murine Ly6). Tumors appear earlier and grow faster when LAN-1 is expressing rat CD59.

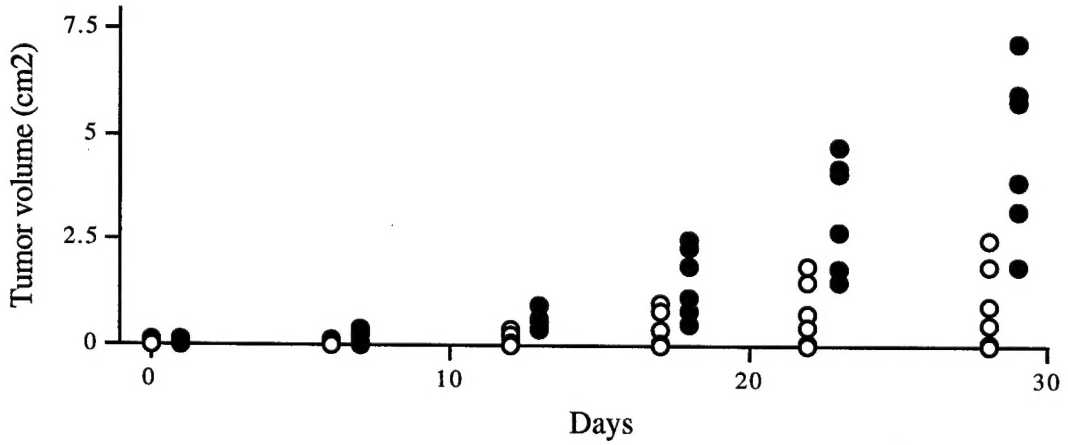

Fig. 3. Growth of human LAN-1 tumor cells in nude rats. Closed circles represent LAN-1 cells expressing rat CD59. open circles represent LAN-1 cells expressing murine Ly6 antigen (transfected as control). 4 x 10^6 cells inoculated per rat, SC in flank. 6 rats inoculated in each group (p= 0.003). Similar results (p=0.0045) were obtained in a second experiment.

Discussion

An important role for CD59 at promoting tumor growth in a rat model of human neuroblastoma has been established. We were unable to established a similar role CD59 in promoting growth of the MCF7 breast

cancer cell line in rats, but this may be due to the lack of spontaneous activation of complement by MCF7. We will therefore now treat MCF7 with complement activating antibodies and repeat in vivo experiments. We will also try different breast cancer cell lines.

TASK 7: Months 12-30: IN VIVO: Continuation of task 2. Will determine if tumor-specific antibodies can elimate or reduce human breast cancer cell growth in rats (about 30 rats). To confirm role of CD59 and complement in any reduction in tumor growth that is observed, rats will be depleted of complement and re-tested (about 20 rats).

This task is behind schedule. We have not yet completed in vitro studies to determine the most suitable anti-tumor complement activating antibodies to use for in vivo studies. We have encountered some problems with MUC1 expression and recognition by anti-MUC1 antibodies (desribed in task 4).

TASK 8: Months 18-36: IN VIVO: Continuation of tasks 3 and 4. Tumors will be grown in rats using cells described above. Will determine whether anti-CD59 antibodies can be targeted to tumors using tumor-specific antibodies and biotin-avidin bridges.

This task has not yet been initiated. The in vitro data described above (task 3) indicate that targeting CD59 on syngeneic 13762 cells in rats will not be effective at sensitizing these cells to complement in vivo. We will investigate other syngeneic rat tumor models for suitability. An alternative approach will be to target rat Crry heterologously expressed on human cancer cells. Anti-MUC1 or anti HER2 targeting to MCF7 or BT474 cells, respectively, may make a suitable model system, and evaluation is underway (above).

KEY RESEARCH ACCOMPLISHMENTS

- The expression of either rat or mouse CD59 on breast human breast cancer cells protects them from lysis by rat and mouse complement, respectively.
- Determined species selectivity of human, rat and CD59. Important for establishing human models of human cancer in rodents for the study of complement.
- Identified the individual residues that confer human CD59 species selective activity. This data is an important step toward identifying the three dimensional structure of the CD59-C9 peptide ligand complex and may assist in design of CD59 inhibitors.
- Have established that CD59 expressed on the surface of a tumor cell can significantly promote tumor growth

REPORTABLE OUTCOMES

Abstracts:

Caragine T., Chen, S., Frey, A.F., and Tomlinson, S. (1998). Protection of human breast cancer cells from anti-MUC1 directed complement-mediated lysis by expression of heterologous CD59. "Antibodies", symposium by Cancer Research Institute.

Caragine, T., Chen, S., Frey, A.F., and Tomlinson, S. (1998) Expression of heterologous CD59 and Crry protects human breast cancer cells from anti-MUC1 directed complement-mediated lysis. Mol. Immunol, 35, p337.

Published papers:

Yu, J., Caragine, T,. Chen, S., Morgan, B. P., Frey, A. and Tomlinson, S. (1999) Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59. *Clin.Exp.Immunol.* **115**, 13-18.

Zhang, H-f., Yu, J., Bajwa E., Morrison, S. L. Tomlinson, S. (1999) Targeting of Functional Antibody-CD59 Fusion Proteins to a Cell Surface. *J.Clin.Invest.,* **103**, 55-61.

Zhang, H-f., Yu, J., Chen, S., Morgan, B.P., Abagyan, R. and Tomlinson, S. (1999) Identification of the Individual Residues that Determine Human CD59 Species Selective Activity. *J.Biol.Chem.*, **274**, 10969-10974.

Chen, S., Caragine, T., Cheung, N-K., and Tomlinson, S. (1999) Surface antigen expression and complement susceptibility of differentiated neuroblastoma clones. *Am.J.Pathol. (undergoing revision)*

CONCLUSIONS

In conclusion, our data strengthen the hypothesis that the modulation of CD59 activity on a tumor cell surface will provide an effective therapy when combined with complement-activating anti-tumor antibodies. Neutralization of CD59 (or other complement regulatory proteins) may also enhance a normally ineffective cytolytic humoral immune response. We have begun to established suitable rodent models and to evaluate the role of complement and CD59 in the growth and control of cancer in vivo. We have had some setbacks with regard to breast cancer specific models in rats, but data using a human a neuroblastoma cell line in rats has established that CD59 can promote tumor growth. These results are likely to be relevant to many types of tumor. We are currently developing improved reagents to proceed with models of breast cancer.

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Clin Exp Immunol 1999; **115**:13–18

Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59

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(Accepted for publication 10 September 1998)

SUMMARY

CD59, decay accelerating factor (DAF) and membrane cofactor protein (MCP) are widely expressed cell surface glycoproteins that protect host cells from the effects of homologous complement attack. Complement inhibitory activity of these proteins is species-selective. We show that the human breast cancer cell line MCF7 is relatively resistant to lysis by human complement, but is effectively lysed by rat or mouse complement. CD59, DAF and MCP were all shown to be expressed by MCF7. The species-selective nature of CD59 activity was used to demonstrate directly the effectiveness of CD59 at protecting cancer cells from complement-mediated lysis. cDNAs encoding rat and mouse CD59 were separately transfected into MCF7 cells, and cell populations expressing high levels of the rodent CD59 were isolated by cell sorting. Data show that rat and mouse CD59 were highly effective at protecting transfected MCF7 cells from lysis by rat and mouse complement, respectively. Data further reveal that rat CD59 is not effective against mouse complement, whereas mouse CD59 is effective against both mouse and rat complement. These studies establish a model system for relevant *in vivo* studies aimed at determining the effect of complement regulation on tumourigenesis, and show that for effective immunotherapy using complement-activating anti-tumour antibodies, the neutralization of CD59 and/or other complement inhibitory molecules will probably be required.

Keywords CD59 complement breast cancer anti-tumour antibody

INTRODUCTION

Complement is one of the major effector mechanisms of the immune system and its activation results in the formation of the C3/C5 convertases, which cleave C5 to initiate the formation of the membrane attack complex (MAC or C5b-9). The cytolytic MAC is formed from the sequential assembly of the soluble plasma proteins C5, C6, C7, C8 and C9. Complement activation on host cells is controlled by various membrane proteins which inhibit C3/C5 convertase formation: decay-accelerating factor (DAF), membrane cofactor protein (MCP) and complement receptor 1 (CR1). Control of cytolytic MAC formation (the terminal complement pathway) on host cell membranes is provided by CD59, a widely distributed cell surface glycoprotein that binds to C8 and C9 in the assembling MAC. For review of complement-inhibitory membrane proteins, see [1].

CD59 and usually DAF and/or MCP are expressed by virtually all breast and other primary tumours and tumour cell lines that have been examined, and several studies have reported the up-regulation of complement-inhibitory proteins on tumour cells [2–8]. Neutralization of complement regulatory proteins on the surface of tumour cells by antibodies significantly increases their susceptibility to complement-mediated lysis *in vitro* [2,3,5,9,10]. The only relevant *in vivo* experiment reported to date shows that pretreatment of rat tumour cells with an antibody that blocks the function of a rat complement inhibitor (Crry/p65), substantially increases survival time of recipient rats after transplantation of treated tumours [11]. There is thus very good evidence to support the hypothesis that tumour-expressed complement inhibitory proteins play an important role in promoting tumour growth by inhibiting complement activation and cytolysis. A significant contributing factor in the lack of success of complement-activating MoAbs in clinical trials to date may therefore be the presence of complement inhibitors on the tumour cell surface. Also, inhibition of tumour-expressed complement regulators may enhance an ineffective cytolytic humoral immune response against tumour cells in therapy which does not involve administration of exogenous activator antibodies.

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An important feature of membrane complement regulatory proteins is their species-selective inhibitory activity [12–18]. These proteins display significant variations in their effectiveness at inhibiting heterologous complement. Thus, the role of complement inhibitors expressed on human cancer cells is difficult to assess in rodent models, since human inhibitors may have limited function against rodent complement. Here we demonstrate directly the protective role that CD59 provides to a human breast cancer cell. We have determined patterns of species-selective activity of endogenous human complement inhibitors, and of rat and mouse CD59 expressed on a human tumour cell line MCF7. These data will permit the planning of relevant *in vivo* studies aimed at determining the role of CD59 in promoting tumour growth.

MATERIALS AND METHODS

Cells and DNA

The human breast cancer cell line MCF7 was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Eagle's modified essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), 0·1% non-essential amino acids and bovine insulin (10 μg/ml). cDNA encoding rat [19] and mouse [20] CD59 was subcloned into the mammalian expression vectors pCDNA3 (Invitrogen, Carlsbad, CA) and pDR2Ef1a [21], respectively. pDR2Ef1a was a gift from Dr I. Anegon (Nantes, France). Stably transfected MCF7 cell populations were selected following the cultivation of cells in the presence of G418 (pCDNA3) or hygromycin (pDR2Ef1a).

Antibodies and complement

Rabbit antiserum to MCF7 cell membranes that was used to sensitize MCF7 cells to complement was prepared by standard techniques [22]. Flow cytometric analysis of MCF7 cells using anti-MCF7 antiserum gave a positive signal at a dilution of 1:200. Cell membranes were prepared by Dounce homogenization of cells in hypotonic media (10 mM sodium phosphate pH 8) and subcellular fractionation to remove nuclei and mitochondria. Anti-rat CD59 MoAb 6D1 [23], anti-mouse CD59 polyclonal antibody [20] and anti-DAF MoAb 1A10 [24] were described previously. Anti-MCP MoAb M75 [25] and anti-human CD59 MoAb YTH53.1 [26] were gifts from Drs D. Lublin (St Louis, MO) and H. Waldmann (Oxford, UK), respectively. FITC-conjugated antibodies used for flow cytometry were purchased from Sigma (St Louis, MO). Normal human serum (NHS) was obtained from the blood of healthy volunteers in the laboratory. Mouse serum was prepared from the blood of BUB/BnJ mice (Jackson Labs, Bar Harbor, ME). Mouse blood was collected by heart puncture, and sera processed after clotting for 3 h on ice. Freshly collected rat serum was purchased from Cocalico Biologicals (Reamstown, PA). All sera were stored in aliquots at −70°C until use.

Transfection of MCF7 cells and flow cytometry

cDNA constructs were transfected into 50–75% confluent MCF7 cells using Lipofectamine according to the manufacturer's instructions (Gibco BRL, Grand Island, NY). Stable populations of MCF7 cells were isolated by three rounds of cell sorting using anti-rat CD59 or anti-mouse CD59 antibodies as described [27]. Analysis of cell surface protein expression was performed by flow cytometry using appropriate antibodies [27].

Cell lysis assays

Complement-mediated cell lysis was determined by both ^{51}Cr release [28] and by microscopic examination following trypan blue staining [29] as described. Both methods gave similar results. Briefly, MCF7 cells were detached by a 3-min/25°C treatment with trypsin/EDTA (Gibco), washed once and resuspended in EMEM/10% heat-inactivated FCS. For the trypan blue exclusion assay, cells were resuspended to 1×10^6/ml. For ^{51}Cr release assay, cells were preloaded at a concentration of 1×10^7/ml (2 h/37°C), washed in complete media and resuspended to 1×10^6/ml. Rabbit anti-MCF7 cell membrane antiserum diluted in EMEM/10% FCS was added and the cells incubated on ice for 30 min. Cells were centrifuged and resuspended to 1×10^6/ml in EMEM/10% FCS. Equal volumes of cells and serum dilutions were incubated for 60 min at 37°C, and cell lysis determined. The effect of anti-rat CD59 MoAb 6D1 on rat complement-mediated lysis was performed as previously described [29].

RESULTS

Lysis of MCF7 cells by human and heterologous serum

Rabbit antiserum raised against MCF7 cell membranes effectively sensitized MCF7 cells to lysis by rat and mouse complement. However, antibody-sensitized MCF7 cells were significantly more resistant to lysis by human complement (Fig. 1). At a concentration of rat serum giving half-maximal lysis, the equivalent human serum concentration resulted in five-fold less lysis. An IgM MoAb directed against the breast cancer-associated antigen MUC1 (BC3 [30]) also sensitized MCF7 cells to lysis by rat complement, but was less effective than the polyclonal antiserum (data not shown).

Expression of endogenous membrane complement inhibitors on MCF7

The relative sensitivity of MCF7 to lysis by rodent, but not human complement, is indicative of species-selective complement

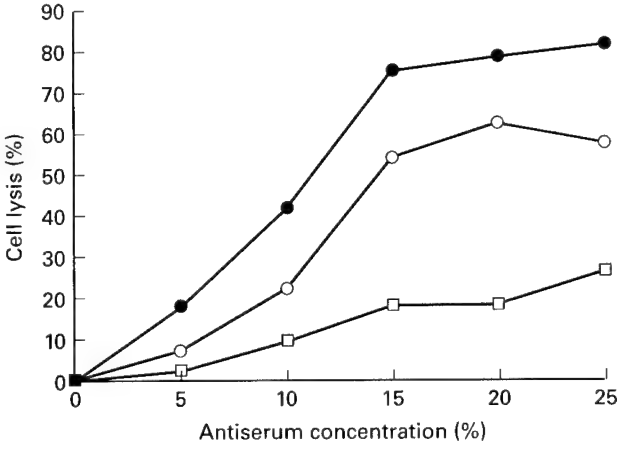

Fig. 1. Complement-mediated lysis of MCF7 cells. MCF7 cells were sensitized to complement by preincubation in the indicated concentrations of anti-MCF7 membrane rabbit antiserum. Sensitized cells were washed in media, exposed to 25% of either human, rat or mouse complement (37°C/60 min), and cell lysis determined. The omission of either sensitizing antibody or of serum in cell lysis assays resulted in a background lysis of < 10% of test value. Figure shows representative data from three separate experiments. ●, Rat serum; ○, mouse serum; □, human serum.

inhibition by endogenous membrane-bound inhibitors. Flow cytometric analysis confirmed the expression of the membrane-bound complement inhibitors CD59, DAF and MCP on MCF7 cells (Fig. 2). Previous data have shown that human CD59 does not function effectively against rat complement [18], and the data shown here indicate that endogenous expression of DAF and MCP on MCF7 does not effectively protect the cells from lysis by rat and mouse complement (Figs 1 and 2).

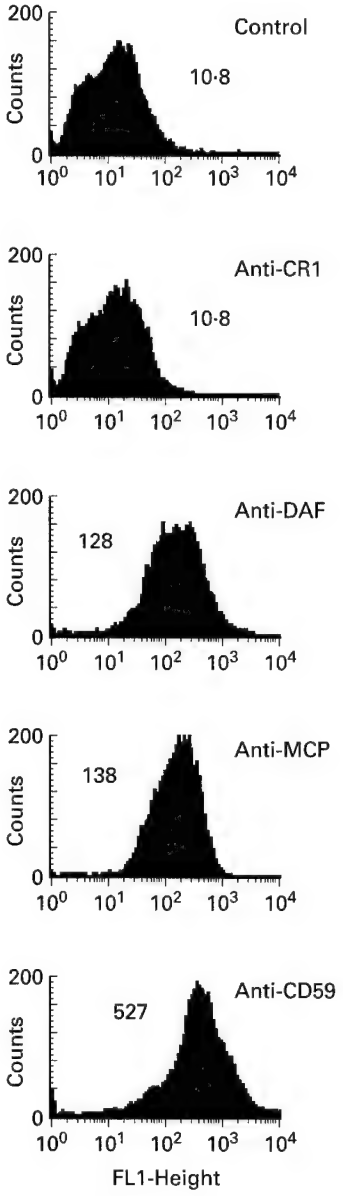

Fig. 2. Endogenous expression of complement-inhibitory proteins by MCF7. Cells were stained by immunofluorescence using MoAbs to human CD59 (YTH53.1), MCP (M75), DAF (1A10), and CR1 (57F) as primary antibodies. Isotype-matched antibodies of irrelevant specificity were used as controls. Relative fluorescence resulting from all control antibodies was <12. Staining with a representative control antibody is shown. Histograms of the relative mean fluorescence intensities are shown.

Complement-mediated lysis of MCF7 cells expressing rodent CD59

The demonstration that heterologous (non-human) cells transfected with human CD59 display increased resistance to lysis by human complement provided direct and unequivocal evidence that human CD59 inhibits human complement-mediated cell lysis [31,32]. The phenomenon of species-selective activity allowed us to use a reciprocal approach to determine directly the functional significance of CD59 expressed on human breast tumour cells.

MCF7 were transfected with rat or mouse CD59 cDNA, and cell populations stably expressing high levels of recombinant rodent CD59 were isolated by cell sorting (Fig. 3). Transfected cell populations were then tested for their susceptibility to complement-mediated lysis to determine whether expression of rodent CD59 correlated with increased resistance to rodent complement. Untransfected MCF7 cells were relatively resistant to lysis by homologous human complement, but were effectively lysed by both rat and mouse complement (Figs 1 and 4). The expression of either rat or mouse CD59 on MCF7 cells, however, protected them from lysis by rat and mouse complement, respectively (Fig. 4). MCF7 cells expressing rat CD59 were almost totally resistant to lysis by 40% rat complement. The increased rat complement resistance of rat CD59-transfected MCF7 cells was reversed by the addition of anti-rat CD59 blocking MoAb 6D1 (not shown), thus confirming that the heterologously expressed rodent CD59 is responsible for providing the observed protection from rodent complement-mediated lysis. It is possible that an anti-CD59 antibody could increase cell lysis by fixing complement, but it has been

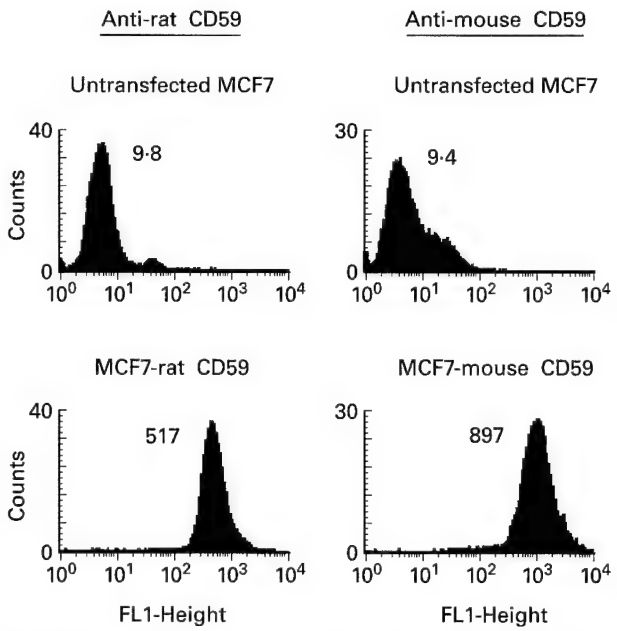

Fig. 3. Expression of rodent CD59 by transfected MCF7. Stably transfected homogenous populations of MCF7 cells expressing either rat or mouse CD59 were isolated by several rounds of cell sorting. Figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using anti-rat CD59 MoAb (6D1) or rabbit anti-mouse CD59 polyclonal antibody. Note that immunofluorescence is not quantitative relative to the different CD59 proteins. Histograms of the relative mean fluorescence intensities are shown.

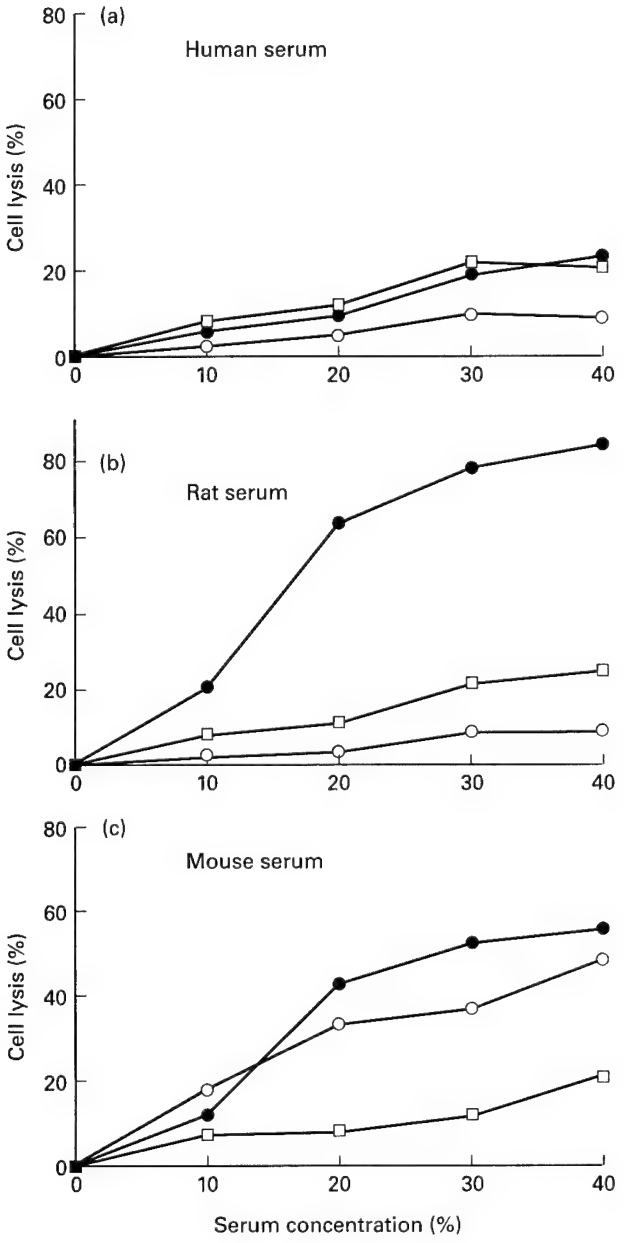

Fig. 4. Complement-mediated lysis of MCF7 cells expressing rodent CD59. Control and transfected MCF7 cells were sensitized to complement by preincubation in 15% anti-MCF7 antiserum. Sensitized cells were exposed to different concentrations of either human (a), rat (b) or mouse (c) serum, and lysis determined. The omission of either sensitizing antibody or of serum in cell lysis assays resulted in a background lysis of < 10% of test value. Figure shows representative data from three different experiments. ●, MCF7; ○, MCF7-rat CD59; □, MCF7-mouse CD59.

demonstrated previously that MoAb 6D1 alone does not cause increased complement-mediated cell lysis by activating complement [23].

Figure 4 further reveals a pattern of species-selective activity for rat and mouse CD59. Rat CD59 effectively protected MCF7 cells from lysis by rat complement (Fig. 4b), but not mouse complement (Fig. 4c). Mouse CD59, on the other hand, was effective against both mouse and rat complement (Fig. 4b,c). The data further indicate that rat, but not mouse CD59 is effective against human complement, since only transfectants expressing rat CD59 showed an increase in resistance to human complement (Fig. 4a). These data demonstrate the relative activities of each CD59 protein against heterologous sera, and data are relevant to establishing rodent models for the study of complement and complement inhibitors in tumour growth and control.

DISCUSSION

The phenomenon of homologous restriction, whereby cells are largely resistant to lysis by homologous complement, is due principally to the species-selective function of CD59 and other membrane complement inhibitors [1]. However, species-selective recognition of complement ligands is not absolute, and CD59 from different species vary in their effectiveness at inhibiting heterologous complement [12–14,17,18,29]. We show that human CD59, which is expressed on virtually all primary tumours and tumour cell lines that have been examined, is not effective against rat or mouse complement. We make use of this finding to demonstrate unequivocally that CD59 expressed on a human breast cancer cell provides efficient protection from complement-mediated lysis. Previous *in vitro* studies have shown that antibodies directed against complement regulatory proteins enhance susceptibility of tumour cells to complement-mediated lysis, and that isolated CD59 protects heterologous erythrocytes from human serum [2,3,5,9]. However, these studies do not exclude the possibility that other antibody- or CD59-interacting membrane molecules may affect complement function at the cell surface [31]. It is also possible that CD59 may provide functions other than direct protection from complement, and some data suggest a role for CD59 in cell signalling [33–35].

Previous *in vitro* data indicate that CD59 also provides cells with protection from the effects of sublytic MAC deposition [36]. Complement activation and sublytic MAC deposition on host cells can trigger the release of various proinflammatory mediators, and can promote the expression of membrane vascular adhesion molecules involved in leucocyte recruitment [37–39]. These inflammatory processes may also play a role in host defence against tumour cells, and promoting their induction may further potentiate the effectiveness of immunotherapeutic approaches based on blocking CD59 function.

Our data indicate that endogenous CD59 expressed on human tumour cells implanted into rodents is unlikely to provide effective protection against complement attack when tumours are targeted by complement-activating antibodies. The relative ineffectiveness of human CD59 against rat and mouse complement presents a serious hindrance to studies aimed at determining the protective role of CD59 (and other complement inhibitors) in rodent hosts bearing human cancers. The current data establish the feasibility of using human cancer cells expressing rodent CD59 to show, *in vivo*, the regulatory effects of CD59 on complement-mediated tumour cell lysis. The aims of this study did not require that cell surface expression of rodent and (endogenous) human CD59 be quantified relative to each other, although quantitative determinations of the activities of the various CD59 proteins against heterologous sera may provide insight into structure/function relationships of CD59 [18].

It is now clear that antibodies against cancer-specific and over-expressed antigens are produced by patients [40]. However, identified endogenous anti-tumour antibodies do not appear to result in tumour destruction, although deposition of complement

may occur. Considered together with the high level of CD59 expression in primary tumours, it is reasonable to postulate that autologous anti-tumour antibodies elicited during tumour growth activate complement on some tumour surfaces, but that tumour cell lysis is prevented by tumour-expressed complement inhibitors. Consequently, progressive tumour growth occurs. Inhibiting complement-inhibitory proteins on a tumour cell surface may enhance the outcome of an endogenous tumour-specific cytolytic humoral immune response, and may also greatly improve the outcome of anti-tumour immunotherapy using complement-activating MoAbs directed against a tumour antigen.

The targeted neutralization of CD59 on tumour cells *in vivo* presents a challenge, since CD59 is widely expressed by normal tissue. Approaches for inhibiting complement inhibitors include the use of humanized antibodies that block function, or high-affinity inhibitory-peptide mimetics. Possible methods for targeting and delivery include the use of encapsulated immunoliposomes or tumour-specific antibodies in techniques utilizing bispecific recognition of CD59 and tumour antigen [41,42]. Recently, the functional targeting of anti-CD59 antibodies to cancer cells by linking them with anti-tumour antibodies was demonstrated *in vitro* [43,44].

In conclusion, our data strengthen the hypothesis that the modulation of CD59 activity on a tumour cell surface will provide an effective therapy when combined with complement-activating anti-tumour antibodies. Neutralization of CD59 (or other complement-regulatory proteins) may also enhance a normally ineffective cytolytic humoral immune response. These hypotheses now need to be tested *in vivo*. To this end, the current data define important parameters necessary for establishing rodent models designed to evaluate the role of complement and CD59 in the growth and control of human cancer.

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Identification of the Individual Residues That Determine Human CD59 Species Selective Activity*

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Formation of the cytolytic membrane attack complex of complement on host cells is inhibited by the membrane-bound glycoprotein, CD59. The inhibitory activity of CD59 is species restricted, and human CD59 is not effective against rat complement. Previous functional analysis of chimeric human/rat CD59 proteins indicated that the residues responsible for the species selective function of human CD59 map to a region contained between positions 40 and 66 in the primary structure. By comparative analysis of rat and human CD59 models and by mutational analysis of candidate residues, we now identify the individual residues within the 40–66 region that confer species selective function on human CD59. All nonconserved residues within the 40–66 sequence were substituted from human to rat residues in a series of chimeric human/rat CD59 mutant proteins. Functional analysis revealed that the individual human to rat residue substitutions F47A, T51L, R55E, and K65Q each produced a mutant human CD59 protein with enhanced rat complement inhibitory activity with the single F47A substitution having the most significant effect. Interestingly, the side chains of the residues at positions 47, 51, and 55 are all located on the short single helix (residues 47–55) of CD59 and form an exposed continuous strip parallel to the helix axis. A single human CD59 mutant protein containing rat residue substitutions at all three helix residues produced a protein with species selective activity comparable to that of rat CD59. We further found that synthetic peptides spanning the human CD59 helix sequence were able to inhibit the binding of human CD59 to human C8, but had little effect on the binding of rat CD59 to rat C8.

Complement activation can lead to the formation of the proinflammatory and cytolytic complement membrane attack complex (MAC)[1] (4) (or C5b-9) on cell membranes, and inappropriate MAC formation on host cell membranes has been implicated in the pathogenesis of various autoimmune and inflammatory diseases. Host cells are normally protected from the effects of the MAC by CD59, a widely distributed membrane-bound glycoprotein.

The mature CD59 protein consists of 77 amino acids arranged in a single compact cysteine-rich domain composed of two antiparallel β-sheets, five protruding surface loops, and a short helix (1, 2). CD59 functions by binding the terminal complement proteins C8 and C9 in the assembling MAC and interfering with its membrane insertion (3–6). Because of species selective recognition of C8 and/or C9 (3, 7), the activity of CD59 is species restricted. However, species restriction is not absolute, and the effectiveness of CD59 from different species against heterologous complement varies.

Mutational analysis of CD59 has begun to define residues important for its complement inhibitory function. Two basic strategies have been used. In one approach, mutagenesis of human CD59 was used to determine protein regions and amino acids essential for its inhibitory function against human complement (8–10). These studies have putatively mapped the human CD59 active site to one side of the protein that contains the short helix. Most of the identified functionally important human residues are well conserved between species and are located in the vicinity of a hydrophobic cleft on the membrane-distal face of the protein (8). In a second related approach, residues important for species selective function have been identified by functional analysis of chimeric human/animal CD59 proteins (11, 12). It is not clear whether CD59 from different species share a common ligand binding site with species selective binding determined by other residues via indirect or allosteric mechanisms, or whether the residues involved in CD59 species selectivity are directly involved in ligand binding.

In a quantitative study on the species selectivity of human and rat CD59, it has been shown that human CD59 is not effective against rat complement, but that rat CD59 is equally effective against rat and human complement (11). Functional analysis of human/rat CD59 chimeric proteins has indicated that the residues responsible for the species selective activity of human CD59 lie between positions 40 and 66 in the primary structure (11). Consistent with this conclusion, a more recent study using chimeric human/rabbit CD59 indicated that sequence between residues 42 and 58 determine human CD59 species selectivity (12). In the current study, we identify individual residues involved in the species selective function of human CD59.

EXPERIMENTAL PROCEDURES

Materials—Human CD59 cDNA was a gift from H. Okada (Nagoya City University, Nagoya, Japan) and the isolation of rat CD59 was described previously (13). The mammalian expression vector pCDNA3 containing the G418 selection marker (Invitrogen, Carlsbad, CA) was used for all DNA manipulation and recombinant protein expression. All DNA primers used in PCR-based mutagenesis procedures were synthesized by Integrated DNA Technologies. Inc. (Coralville, IA). Recombi-

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[1] The abbreviations used are: MAC, membrane attack complex; CHO, Chinese hamster ovary; PCR, polymerase chain reaction.

nant CD59 proteins were expressed in Chinese hamster ovary cells (CHO) that were maintained in Dulbecco's modified essential medium containing 10% heat-inactivated fetal calf serum. Rabbit antiserum to CHO cell membranes (14) was prepared as described (15). Anti-tag monoclonal antibody 2A10 directed against NANPNANPNA, a repeat domain of *Plasmodium falciparum* circumsporozoite protein, was described previously (4). Fluorescein isothiocyanate-conjugated antibodies used for flow cytometry were from Sigma. Rat C8 was purified as described (16). Recombinant soluble rat and human CD59 was expressed in CHO cells and purified by affinity chromatography as described (2). Human C8 was purchased from Advanced Research Technologies (San Diego, CA). Four CD59 sequence specific peptides were synthesized and high pressure liquid chromatography-purified (>80%) by Genemed (South San Francisco, CA); peptide 1, RLRENELTY; peptide 2, FNDVTTRLRENELTY; peptide 3, WKFEHCNFNDVTTRLRENELTY; and peptide 4, NFNDVTTRLRE. Normal human serum was obtained from the blood of healthy volunteers in the laboratory. Rat serum was purchased from Cocalico Biologicals (Reamstown, PA).

Construction of Mutant CD59 Proteins—Residue substitutions in human CD59 were prepared by standard PCR mutagenesis techniques as described (8, 11). In the first PCR amplification, 5′ and 3′ primers matching an untranslated region of human CD59 and containing a *Hin*dIII and *Apa*I site, respectively, were paired with primers spanning the target site in which a rat amino acid codon was substituted. Each final PCR product was digested with *Hin*dIII and *Apa*I and was cloned into pCDNA3 expression vector for sequencing and expression. To quantitate the relative expression of recombinant proteins, an oligonucleotide encoding the tag-peptide sequence NANPNANPNA was inserted after the human CD59 N-terminal Leu codon as described (8).

Expression of Recombinant Proteins—CHO cells were transfected with pCDNA3 constructs using LipofectAMINE™ according to the manufacturer's instructions (Life Technologies, Inc.). Stable transfectants were selected by the addition of G418 (400 μg/ml) 3 days after transfection. After 14 days of selection, stable populations of CHO cells each expressing similar levels of tagged recombinant protein were sorted by flow cytometry by means of anti-tag monoclonal antibody 2A10 as described (8). At least three rounds of cell sorting were required to obtain homogeneous cell populations expressing similar levels of recombinant protein.

Flow Cytometry—For quantitative analysis of tagged recombinant protein expression, stably transfected detached CHO cells were incubated with monoclonal antibody 2A10 (10 μg/ml) for 30 min at 4 °C. Cells were then washed, and incubated with fluorescein-conjugated anti-mouse IgG for 30 min at 4 °C. Cells were then washed again, fixed with 2% paraformaldehyde in phosphate-buffered saline, and analyzed using a Becton Dickinson FACScan. All incubations and washing were carried out in Dulbecco's modified essential medium, 10% fetal calf serum. Cells for sorting were fluorescently labeled as above but were not fixed. Sorting was done in a Coulter Epics Elite with EPS sort module (Coulter Corp., Miami, FL).

Cell Lysis Assay—Complement-mediated CHO cell lysis assays were performed as described previously (8). Briefly, cells were incubated in 20% heat-treated anti-CHO antiserum, washed once, and exposed to 20% human or rat serum (either active or heat-inactivated). Cell lysis was determined by both trypan blue exclusion and by measuring the release of a preloaded fluorescent probe, calcein-AM (8). Both methods gave similar results. Lysis was determined using sets of homogenous cell populations expressing similar levels of rat CD59, human CD59, or chimeric CD59 on their surface (Ref. 11 and also see above).

CD59 Binding Assay—The ability of synthetic human CD59 peptides to inhibit the binding of CD59 to its ligand C8 was determined using a previously described microtiter plate binding assay (4). Briefly, human or rat C8 was coated onto microtiter wells, and the respective binding of biotinylated human or rat CD59 was determined in the presence of varying concentrations of peptide. CD59 (at final concentration of 20 μg/ml in phosphate-buffered saline containing 0.1% bovine serum albumin) and different concentrations of peptide were mixed before addition to C8 coated wells. All peptides were prepared as a 4 mg/ml stock solution in phosphate-buffered saline. Peptide 3 (see above) required a short sonication for solubilization. Binding of biotinylated CD59 was determined by means of Extravidin-peroxidase soluble *o*-phenylenediamine substrate system (Sigma). CD59 was biotinylated using EZ-link LC-biotin as described by the manufacturer for the biotinylation of IgG, using the same protein:biotin ratios (Pierce). Ratios were calculated based on molecular weights of 18,000 and 155,000 for CD59 and IgG, respectively (these ratios were determined to be important).

Molecular Modeling—Modeling by homology and subsequent analyses were performed with the ICM program developed for molecular modeling and structure predictions by global restrained energy optimization of arbitrarily constrained molecules (17, 18). The energy is calculated with ECEPP/3 force field (19) extended by recently developed solvation and side-chain entropic terms (18). The following terms were included in the energy function: van der Waals and 1-4 nonbonded interactions, hydrogen bonding, torsion, electrostatic, disulfide bond restraints, solvation energy, and side-chain entropy. Cut off distance for truncation of van der Waals and electrostatic interactions was set to 7.5, and for hydrogen bond interactions it was set to 3.0. The side-chain torsion angles were predicted by simultaneous global optimization of the energy for all residues that were different in the rat and human sequences. The biased probability Monte Carlo-minimization method (18) was used for global optimization. A region around insertion at the C terminus was predicted with the loop prediction procedure described earlier (20). The final root mean square deviation of the backbone atoms between the human and rat coordinate sets was 0.64A. The molecular surface was built with the fast analytical "contour buildup" algorithm (21).

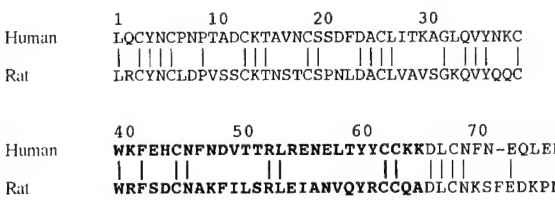

FIG. 1. **Sequence alignment of human and rat CD59.** The 40–66 sequence previously determined to contain the residues important for conferring species selective function (11) are shown in *bold*. Identical residues are indicated by *vertical bars*. Mature protein sequences are shown and the C-terminal end of rat CD59 is predicted.

RESULTS

Molecular Modeling and Comparative Analysis of Human and Rat CD59—Human CD59 is not an effective inhibitor of rat complement, and previous functional analysis of chimeric human/rat CD59 proteins demonstrated that residues important for the species selective function of human CD59 lie between positions 40–66 in the primary structure (11). To identify the individual residues involved in human CD59 species selective function, we first built a model of rat CD59 on the basis of the known structure of human CD59 and determined the location and the distribution of nonconserved surface patches within the 40–66 amino acid region of human and rat CD59. The CD59 sequences can be aligned with 45% sequence identity and only a single residue insertion at the C terminus of the protein (Fig. 1). A model by homology was built and refined using the ICM global energy optimization procedure (see "Experimental Procedures"). Analytical molecular surfaces (21) for both human and rat proteins were then built, and the surface shape and distribution of surface patches of human and rat CD59 were analyzed (see Fig. 2, *A* and *B*). Residues 40–66 are shown in color in Fig. 2, and as previously shown (11), they all map to one side of the molecule. The region of CD59 shown in white represents the three-dimensional location of the regions that do not appear to be involved in species selective function (*i.e.* residues 1–39 and 66–77).

Comparison of the models revealed pronounced differences between groups of clustered residues within the 40–66 region. The two largest and most conspicuously different groupings were six clustered residues at positions 48, 52, 55, 56, 57, and 58 that will produce very different surface patterns of electrostatic potential and hydrophobicity in the human and rat proteins, and three residues at positions 41, 43, and 44 that form a cluster of difference in shape and electrostatic properties. Other nonconserved groupings include residues 47 and 51, and residues 60, 62, and 66 that occur in a linear arrangement across the membrane proximal face of CD59 (this arrangement is not apparent in the views of CD59 shown in Fig. 2).

Mutational Analysis of CD59—Human to rat amino acid

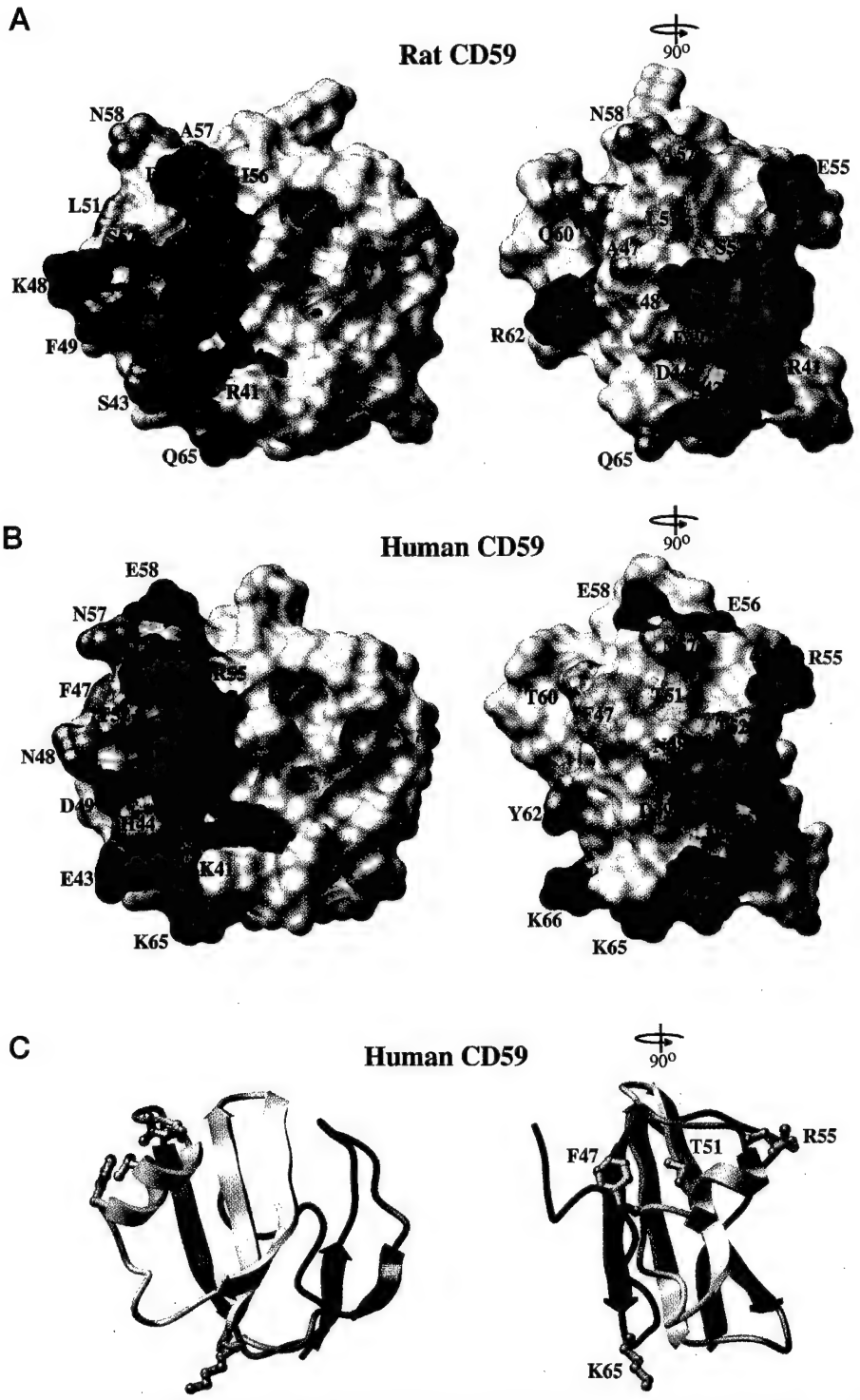

FIG. 2. **Diagrams of human and rat CD59.** *Panels A* and *B* show a comparison of molecular surfaces in the region identified as important for species selective function (residues 40–66). The surface of regions 1–39 and 66–77 that are not important for species selectivity is colored *white*. Conserved residues previously identified as functionally important in human CD59 (8, 9) are colored *magenta* and are not numbered. Side chains of all nonidentical residues within the 40–66 sequence (and potentially responsible for species selective activity) are colored *red* (negatively charged residues), *blue* (positively charged residues), *yellow* (hydrophobic residues), and *green* (other residues). Backbone atoms of other residues, as well as side chains of residues that are identical in human and rat CD59 and therefore not important for species selectivity, are shown in *white*. *Panel C* is a ribbon diagram of human CD59 showing the residues experimentally determined to influence species selectivity.

substitutions that result in acquisition of rat complement inhibitory activity will identify functionally important residues. To determine whether the candidate residue groups identified by model comparison above are involved in the species selective function of human CD59, the groups of residues were substituted for corresponding rat residues. Some additional residues were also substituted so that all nonidentical residues within the 40–66 sequence were accounted for. Further, some substitutions were made for residues that are outside of the 40–66 sequence, but that neighbor human residues previously identified as important for CD59 activity (8, 9). The mutant human CD59 proteins containing groups of substituted rat residues that were initially prepared and tested are shown in Fig. 3 (mutant series A). The proteins were recombinantly expressed

on the surface of CHO cells, and cell populations expressing similar levels of protein were isolated (see "Experimental Procedures") and then assayed for their susceptibility to human and rat serum. CD59 expression levels were quantitated using flow cytometry by means of an epitope tag inserted at the N terminus of all recombinantly expressed proteins as described previously (8, 11).

The data in Fig. 4 show that human CD59, rat CD59, and all chimeric CD59 proteins are equally effective against human complement, indicating that none of the substitutions had any adverse effect on protein conformation and activity. When compared with the activity of human CD59, the A1, A3, A6, and A7 chimeric proteins provided enhanced protection against rat complement. The A6 and A7 proteins were about 25 and 70% as effective as rat CD59 against rat complement, respectively (calculated based on the difference between rat complement-mediated lysis of CHO cells expressing either human or rat CD59) (Fig. 4). The A6 and A7 proteins were significantly more effective against rat complement than the A1 and A3 proteins. Each of the A6 and A7 chimeras contained only two substituted residues (Fig. 3), putatively identifying one or more human residues from a total of four that primarily determine the species selective activity of human CD59, *i.e.* Phe-47, Thr-51, Arg-55, and Lys-65. The human to rat R55E substitution is common to the A1, A3, and A6 proteins, suggesting that this substitution is responsible for the slightly increased inhibitory activity against rat complement of the A1 and A3 proteins.

In a second series of mutations, each of the four candidate functionally important human residues, and a residue not expected to effect species selectivity (Lys-41), were individually substituted for corresponding rat residues (see Fig. 3, *series B*). The data in Fig. 5 show that the individual substitution of each candidate human residues with the corresponding rat residue produced a protein with enhanced rat complement inhibitory activity. The F47A substitution (B4 mutant) was by far the most effective at enhancing the activity of human CD59 against rat complement. This single rat residue substitution in human CD59 resulted in a protein that was about 65% as effective as rat CD59 against rat complement (Fig. 5). The T51L and R55E substitutions resulted in proteins that each possessed close to 20% of rat CD59 inhibitory activity. The K65Q substitution also appeared to display a small (about 10%), but statistically insignificant increase in activity against rat complement. Nevertheless, the A6 protein that contains both an R55E and K65Q substitution (Fig. 4) was slightly more effective against rat complement than an R55E substitution alone (Fig. 5). The single human to rat residue substitution at position 41 (K41R) did not alter the functional characteristics of human CD59, as predicted from functional data obtained with the A2 protein (contains a K41R substitution). To further confirm an important role for residue 47 in determining the species selective function of human CD59, an additional mutant protein was prepared containing a human to mouse substitution at residue position 47 (protein B5 (F47G), see Fig. 3). We have shown previously that human CD59 is not effective against mouse complement (22), and the single F47G substitution produced a mutant protein possessing species selective function that was quantitatively similar to the F47A (human to rat) substitution (Fig. 5). None of the residue substitutions had any effect on human complement inhibitory activity, indicating that all recombinant proteins were correctly folded (Fig. 5).

The positions and side-chain characteristics of the identified functionally important human residues on the CD59 protein are shown in Fig. 2. Interestingly, the side chains of the Phe-47, Thr-51, and Arg-55 residues are all located in a strip on the same face of the CD59 helix (Fig. 2*C*). In a final mutant CD59 protein, each of the three human helix residues that individually affected species selective function were substituted with rat residues (mutant B7, Fig. 5). The rat complement inhibitory activity of this mutant protein approached that of rat CD59 (about 80% as effective) (Fig. 5), further indicating that the identified helix residues, and in particular Phe-47, are the principal determinants of human CD59 species selective function.

Effect of Synthetic CD59 Peptides on the Binding of CD59 to C8—We used a previously characterized microtiter plate binding assay to determine whether synthetic human CD59 peptides from the vicinity of the helix region could interfere with the binding of CD59 to its ligand, C8. We found two peptides, both spanning the helix residue sequence, that modestly inhibited the binding of human CD59 to human C8; a peptide to

Mutant series A

A1: K38Q, K41R, E43S, H44D, N48K, D49F, T52S, R55E, E56I, N57A, E58N
A2: K38Q, K41R, E43S, H44D
A3: N48K, D49F, T52S, R55E, E56I, N57A, E58N
A4: T60Q, Y62R, K66A
A5: F23L, A31S, L59Q
A6: R55E, K65Q
A7: F47A, T51L

Mutant series B

B1: K41R
B2: F47G
B3: F47A
B4: T51L
B5: R55E
B6: K65Q
B7: F47A, T51L, R55E

FIG. 3. **Human to rat amino acid substitutions made in chimeric CD59 proteins.** The individual residue substitutions shown in series B were selected based on functional data obtained from series A mutant proteins.

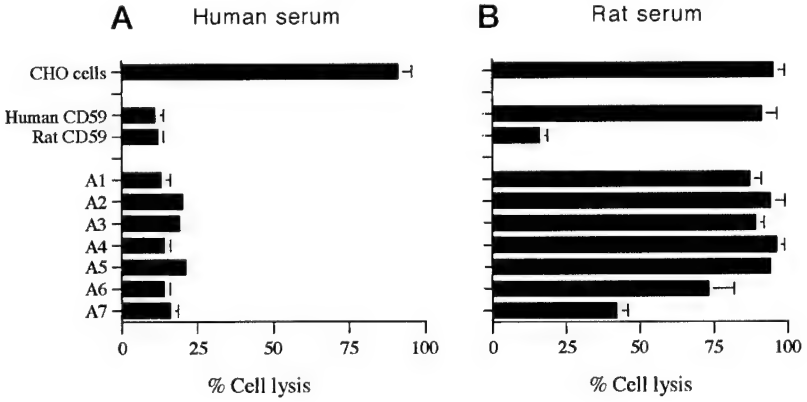

FIG. 4. **Complement resistance of CHO cells expressing human and rat CD59 and human-rat chimeric CD59 proteins.** Stable CHO cell populations expressing similar levels of recombinant protein were exposed to 20% human serum (*panel A*) or rat serum (*panel B*) and lysis percentage determined. A1–A7 represent mutant human CD59 proteins containing groups of rat residue substitutions (refer to Fig. 3). An N-terminal epitope tag that does not effect CD59 function was used to measure cell surface expression of CD59 (8, 11) (also see "Experimental Procedures"). Mean ± S.D. (n = 6).

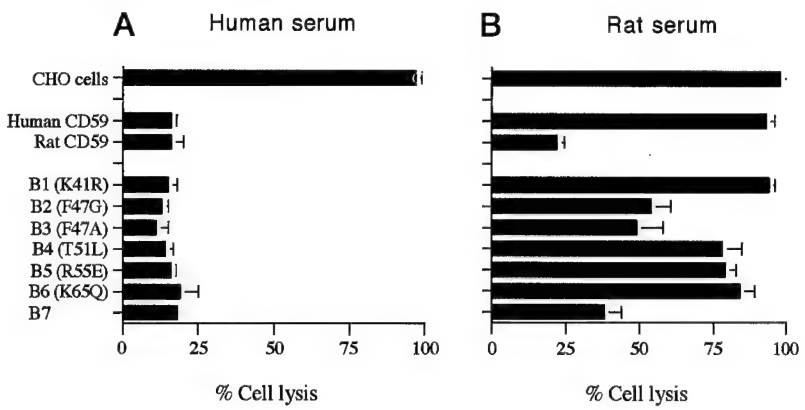

FIG. 5. **Complement resistance of CHO cells expressing human CD59 containing single rat residue substitutions.** Stable CHO cell populations expressing similar levels of recombinant protein were exposed to 20% human serum (*panel A*) or rat serum (*panel B*) and lysis percentage determined. The B7 mutant protein contained three human to rat residue substitutions (F47A, T51L, and R55E, see Fig. 3.) Mean ± S.D. (n = 4).

CD59 ratio of 320:1 inhibited binding by 30–40% (peptides 2 and 3, Fig. 6). In contrast, the same peptides were much less effective at inhibiting the binding of rat CD59 to rat C8. This data is consistent with the above mutagenesis data and suggests that the identified CD59 helix residue(s) are directly involved in the species selective binding of C8. A shorter peptide containing the helix residues (peptide 4, Fig. 6), as well as a peptide containing sequence C-terminal to the helix residues (peptide 1, Fig. 6), had little effect on the binding of CD59 to C8. A possible explanation for the lack of inhibition by the short helix peptide is that the N- and/or C-terminal helix residue extensions of the longer peptides stabilize a structure that is more favorable for binding. In a functional assay, the CD59 peptides were also tested for their effect on human C5b-9-mediated hemolysis of human erythrocytes (as described in Ref. 23). Peptide binding to C8 in the assembling C5b-9 complex at a cell surface might interfere with the inhibitory effect of CD59, but the peptides had no effect on C5b-9-mediated hemolysis (not shown).

DISCUSSION

By measuring the inhibitory activity of chimeric human-rat CD59 proteins against human and rat complement, it was previously determined that the species divergent 40–66 residue sequence contains the residues important for the species restricted function of human CD59 (11). We have now identified individual residues within this region that are responsible for human CD59 species selectivity. Individual substitutions of the human residues Phe-47, Thr-51, and Arg-55 for corresponding rat residues, each produced proteins with enhanced activity against rat complement. The substitution of all three residues in a single protein resulted in a CD59 protein (termed B7) with a species selective activity that was quantitatively similar to that of rat CD59; compared with the negligible activity of human CD59 against rat complement, the B7 mutant was about 80% as effective as rat CD59 at inhibiting rat complement. A fourth residue, Lys-65, also appears to contribute to the selectivity of human CD59 function, albeit to a lesser degree than the three helix residues, and may at least partly account for the slightly reduced activity of the B7 protein against rat complement as compared with rat CD59. No other nonconserved residue within the 40–66 sequence had any detectable effect on the species selective function of human CD59.

The residues identified here as determinants of species selectivity are distinct from previously identified human CD59 active site residues. Site-directed mutagenesis of human CD59 (nonconservative substitution) has indicated that residues Phe-23, Asp-24, Trp-40, Arg-53, Leu-54, Glu-56, and Tyr-62 are important for human CD59 function (8–10). With the exception of Tyr-62, these residues are located on the membrane-distal face of CD59 in the vicinity of a hydrophobic cleft, and with the further exception of residues Phe-23 and Glu-56, they are conserved in human and rat CD59 (refer to Fig. 1). It is possible that CD59 from different species possess a conserved ligand binding site, and that nonconserved residues in CD59 proteins influence the specificity of ligand binding via indirect or allosteric mechanisms. Alternatively, CD59 residues involved in determining the species selectivity may participate directly in ligand binding. We identify Phe-47, Thr-51, and Arg-55 as being the residues primarily involved in restricting human CD59 activity. In rat CD59, these residues are replaced by alanine, leucine, and glutamic acid, respectively. Interestingly, these three residues form a continuous strip parallel to the axis of the CD59 helix and are exposed to the solvent. It is therefore considered unlikely that the side chains of residues Phe-47, Thr-51, and Arg-55 influence specificity through affecting the relative position of the helix with respect to other binding pocket residues. Rather, it seems more likely that these residues are directly involved in ligand binding. The side-chain differences between the three human and rat CD59 residues are such that all three substitutions can potentially contribute to specificity, provided that this phase of the short helix is involved in the direct interaction. The phenylalanine and alanine side chains are both hydrophobic but differ in size, the threonine and leucine differ by a polar group and hydrophobic character, and arginine and glutamic acid have different charges although they share the same hydrophobic stem.

The single residue that contributes by far the most to human CD59 species selective function is Phe-47. A key role for Phe-47 in species selectivity was further indicated by functional analysis of a human to mouse F47G substitution (see "Results"). Perhaps a binding pocket on the rodent C8/C9 ligands that can accommodate the rodent alanine and glycine residues cannot accommodate the large phenylalanine residue in the corresponding location on human CD59. Such an explanation is compatible with the fact that rat (11) and mouse (22) CD59 are both effective against human complement, whereas human CD59 does not function effectively against rodent complement. Also compatible with this "docking" concept is the previous result that a nonconservative F47E mutation resulted in a human CD59 protein with only a weak protective effect against human complement (9). It was suggested from this finding that Phe-47 may be at the periphery of the human CD59 active site. The positively charged residue Lys-65 in human CD59 that is replaced by a polar Gln-65 in rat CD59 also had a small effect on species selectivity. Lys-65 is positioned next to the conserved and functionally important residue Asp-24 (9) and is located at one end of the hydrophobic cleft that may be important for complement ligand binding (see above). The current data does not exclude the possibility that the location of residues that determine species selectivity may differ in different CD59 proteins. Nevertheless, consistent with the current data, a recent analysis of the species selectivity of chimeric human/

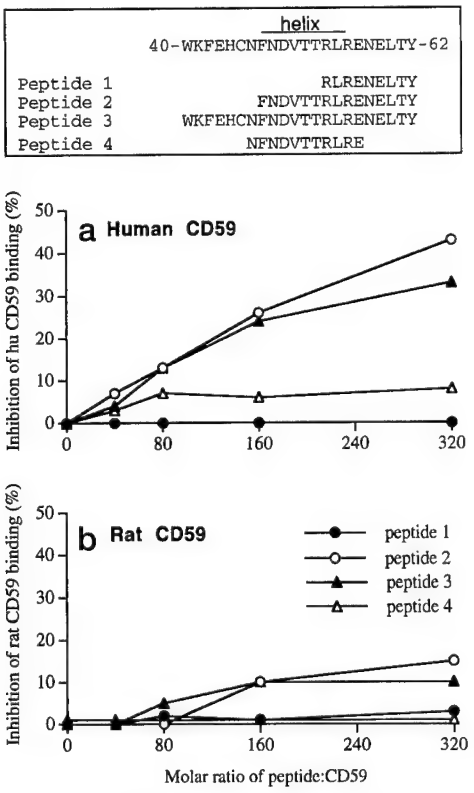

FIG. 6. **The effect of human CD59-specific peptides on the binding of human and rat CD59 to their autologous C8 ligand.** Purified human (*a*) or rat (*b*) C8 was adsorbed onto plastic (microtiter plates), and the binding of biotinylated human (*a*) or rat (*b*) CD59 was determined in the presence of various synthetic human CD59 sequence-specific peptides. The amino acid sequences of the peptides are shown in the *top panel*. The sequences span, or are in the close vicinity of, the helix (residues 47–55) (representative data shown: *panel a*, $n = 3$; *panel b*, $n = 2$).

rabbit CD59 proteins indicated that species selectivity is determined solely by sequence contained between residues 42 and 58 of human CD59 (12).

Analysis of the patterns of species selectivity of various cloned CD59 proteins is consistent with functional contributions from residues at positions 47, 51, and 55. Mouse and rat CD59 display similarities in their pattern of species selectivity with regard to homologous and human complement, and both have a small side chain in position 47 (glycine and alanine, respectively), a long hydrophobic side chain in position 51 (leucine and methionine, respectively), and a conserved residue at position 55 (glutamic acid) (refer to Fig. 1). In contrast, human, primate, and pig CD59 are all effective against human but not rodent complement. In CD59 from these species, the Phe-47 is conserved, the residue substitution threonine to serine in position 51 preserves the side-chain hydroxyl group, whereas human Arg-55 is replaced in primate and pig CD59 by a smaller side chain, rather than to an oppositely charged residue (as in rodent CD59). Rabbit CD59, however, which does not provide effective protection from human complement (12), contains an oppositely charged residue at position 55 (glutamic acid instead of arginine) and may account for its incompatibility with human CD59, despite similarities in positions 47 and 51.

Recombinant soluble complement inhibitors based on membrane regulators of complement activation are effective at suppressing inflammation and disease pathology in a variety of animal models, and an understanding of the molecular basis for CD59 function may provide the rationale for the design of efficient soluble MAC inhibitory constructs for clinical application. Inhibiting the terminal pathway of complement but leaving the activation pathway intact may offer significant clinical advantages in diseases in which the MAC plays an important role. This is because products of the complement activation pathway play important roles in immunity to infection and in immune complex catabolism. Evidence indicates that an effective CD59-based inhibitor will also provide efficient protection from complement-mediated hyperacute rejection of xenotransplanted tissue (24, 25). Transgenic pig organs expressing high levels of human CD59 are protected from human complement and show prolonged survival when transplanted into primates (26, 27). The identification here of the residues important for species selectivity and the conservation of these residues in human and pig CD59 support the view that the level of CD59 expression will be more important than the species of CD59 in prolonging pig to human graft survival (28, 29). Finally, defining the functional site(s) of CD59 may also assist in the design of inhibitors of CD59. Inhibiting CD59 function on the surface of tumor cells may prove effective in anti-tumor complement-dependent immunotherapy.

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Targeting of functional antibody-CD59 fusion proteins to a cell surface

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Complement is involved in the pathogenesis of many diseases, and there is great interest in developing inhibitors of complement for therapeutic application. CD59 is a natural membrane-bound inhibitor of the cytolytic complement membrane attack complex (MAC). In this study, the preparation and characterization of antibody-CD59 (IgG-CD59) chimeric fusion proteins are described. Constructs were composed of soluble CD59 fused to an antibody-combining site at the end of C_H1, after the hinge (H), and after C_H3 Ig regions. The antigen specificity of each construct was for the hapten 5-dimethylaminonaphthalene-1-sulfonyl (dansyl). Correct folding of each IgG-CD59 fusion partner was indicated by recognition with anti-CD59 antibodies specific for conformational determinants and by IgG-CD59 binding to dansyl. The IgG-CD59 fusion proteins all bound specifically to dansyl-labeled Chinese hamster ovary cells and provided targeted cells, but not untargeted cells, with effective protection from complement-mediated lysis. Data indicate that CD59 must be positioned in close proximity to the site of MAC formation for effective function, and that modes of membrane attachment other than glycophosphatidylinositol linkage can affect CD59 functional activity.

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Introduction

Activation of complement via either the classical or alternative pathway results in the generation of C3 convertase, a central enzymatic complex of the complement cascade that cleaves serum C3 into C3a and C3b. The C3b product can bind covalently to an activating surface and can participate in the further generation of C3 convertase (amplification loop). C3 convertases also participate in the formation of C5 convertase, a complex that cleaves serum C5 to yield C5a and C5b. Formation of C5b initiates the terminal complement pathway, resulting in the sequential assembly of complement proteins C6, C7, C8, and $(C9)_n$ to form the membrane attack complex (MAC, or C5b-9).

The complement activation products (particularly C5a and MAC) are powerful mediators of inflammation and can induce a variety of cellular activities, including the release of proinflammatory molecules (1–6). Complement can also cause tissue damage directly, because of membrane deposition of the cytolytic MAC. It is now clear that complement plays an important role in the pathology of many autoimmune and inflammatory diseases, and that it is also responsible for many disease states associated with bioincompatibility, *e.g.*, postcardiopulmonary inflammation and transplant rejection (7–13).

Human cells are normally protected from inappropriate complement activation by various membrane-bound complement inhibitors (14, 15). These molecules include complement receptor 1 (CR1), decay-accelerating factor (DAF), and membrane cofactor protein (MCP), which inhibit the early complement activation pathway and the generation of C3 convertase. CD59 is an inhibitor of the terminal complement pathway. CD59 is a widely distributed 18–21-kDa glycoprotein attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor, and functions by preventing assembly of the terminal lytic MAC.

Recombinant soluble complement inhibitors based on membrane inhibitors of complement have been prepared by the removal of membrane-linking regions. Soluble inhibitors of complement activation function effectively *in vitro*, and their administration to animals in models of disease has been shown to suppress inflammation and disease pathology (16–23). Nevertheless, there are concerns regarding the clinical use of systemic inhibitors of complement activation, because activation pathway products play a crucial role in immunity to infection and immune complex catabolism (24–28). A potential advantage of CD59-based inhibitors over inhibitors of complement activation is that CD59 will block MAC formation but will not affect the generation of C3 and C5 activation products. In this respect, MAC has been implicated in the pathogenesis of several autoimmune and inflammatory diseases (9, 29–35). CD59 may also be clinically useful for providing protection from complement-mediated hyperacute rejection of xenotransplanted tissue. It has been shown that human CD59 and/or DAF expressed on the surface of transgenic pig tissue can considerably prolong the survival of transgenic organs when transplanted into primates (36, 37).

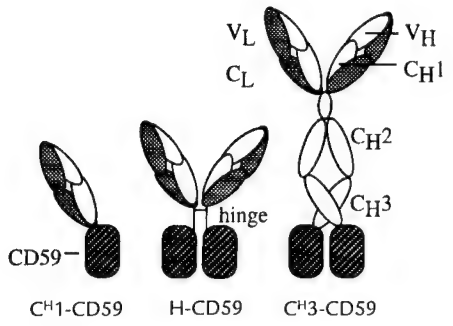

Figure 1
Diagram of antibody-CD59 fusion proteins expected from the expression constructs. Antibody domains are labeled (V_L, variable light; V_H, variable heavy; C_L, constant light; C_H, constant heavy). The specificity of the antibody-combining site (on V_L/V_H domains) is for the hapten dansyl. *Dansyl*, 5-dimethylaminonaphthalene-1-sulfonyl.

Soluble untargeted CD59 is not an effective inhibitor of MAC formation *in vitro* (9), and there are no reports of soluble CD59 being tested *in vivo*. However, because membrane-bound CD59 provides effective intrinsic protection from MAC formation, the targeting of soluble CD59 to a cell membrane and site of MAC formation may enhance its activity. In this study, we attempt to construct an improved complement inhibitory molecule by joining a soluble CD59 unit to various antibody fragments containing antigen-combining sites. In addition to the potential benefits of targeting a complement inhibitor, the joining of different proteins to immunoglobulin γ chains has been shown to increase the half-life of proteins in the circulation and increase binding affinity of the fusion partner due to dimerization by antibody chains. In this feasibility study, antibody fragments specific for the hapten 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) are joined to CD59. Dansyl can be used to label cell surfaces and thus provides a convenient target antigen for *in vitro* studies using antibody-CD59 fusion proteins. We show that various targeted antibody-CD59 fusion proteins, but not untargeted CD59, effectively protect cells against complement-mediated lysis in an antigen-specific manner.

Methods

Cell lines. TWS2 is the immunoglobulin nonproducing mouse myeloma cell line Sp2/0, transfected previously with a light chain construct incorporating murine κ anti-dansyl variable domain joined to human Cκ constant domain (38). TWS2 was cultured in Iscove's Modified Dulbecco's Medium (GIBCO BRL, Grand Island, New York, USA) containing 10% FCS. Chinese hamster ovary (CHO) cells were grown in DMEM supplemented with 10% FCS.

DNA, antibodies, and reagents. CD59 cDNA (39) and anti-CD59 monoclonal antibody (MAB) 1F5 (40) were kindly provided by H. Okada (Osaka University, Osaka, Japan). Anti-CD59 MABs YTH53.1 (41) and P282 were the kind gifts of B.P. Morgan (University of Wales, Cardiff, United Kingdom) and A. Bernard (Hôpital L'Archet, Nice, France), respectively. Anti-CD59 MAB MEM43 was purchased from Harlan Bioproducts for Science (Indianapolis, Indiana, USA). Normal human serum (NHS) was obtained from the blood of healthy volunteers in the laboratory and stored in aliquots at −70°C. Rabbit anti-CHO cell membrane antiserum was prepared by inoculation with CHO cell membranes by standard techniques (42). Anti-dansyl IgG4 was prepared by antigen affinity chromatography as described previously (43).

Construction of antibody-CD59 fusion proteins. cDNA encoding a soluble CD59 functional unit (residues 1–77) (44) was generated by PCR amplification to contain a blunt 5′ end and an Eco R1 site at its 3′ end. The GPI-addition signal sequence of CD59 was deleted in product preparation. The PCR product was blunt-end ligated in frame to the 3′ end of a Ser-Gly encoding spacer sequence ($SG_4SG_4SG_4S$). Using unique restriction sites generated in the human IgG3 heavy-chain constant region (45), the spacer-CD59 sequence was inserted at the 3′ end of various human IgG3 heavy-chain encoding regions. CD59 was inserted (5′-blunt/EcoR1-3′) after the heavy-chain constant region 1 (C_H1-CD59) exon, immediately after the hinge (H) region at the 5′ end of the C_H2 exon (H-CD59), and after the C_H3 exon (C_H3-CD59). For expression, the IgG-CD59 gene constructs were subcloned into the expression vector 4882PAG, which contains the murine heavy-chain anti-dansyl variable region (45, 46). The constant region sequences in the 4882PAG vector were replaced by the IgG-CD59 constructs using unique Bam HI and Sal I sites (45, 46). For the C_H3-CD59 construct, human IgG3 heavy-chain constant region was replaced by human IgG4 (47).

Transfection and clone selection. 4882PAG/IgG-CD59 expression plasmid constructs were transfected into TWS2 cells using lipofectamine, according to the manufacturer's instructions (GIBCO BRL). Three days after transfection, medium containing 1 μg/ml mycophenolic acid, 2.5 μg/ml hypoxanthine, and 42 μg/ml xanthine was added to the cells for selection of stable transfected populations. After 3 weeks in selection medium, transfectoma clones expressing IgG-CD59 proteins were isolated by assaying culture supernatant for IgG-CD59 fusion proteins by ELISA (see below). High-expressing clones were selected by dilution method.

ELISA and protein assays. Detection of IgG-CD59 fusion proteins and their relative concentrations was accomplished using a standard ELISA technique (42). Briefly, microtiter plates were coated with dansylated BSA (see below; 100 μg/ml overnight at 4°C) and then blocked with 2% BSA in PBS. Culture supernatant containing fusion proteins or purified sam-

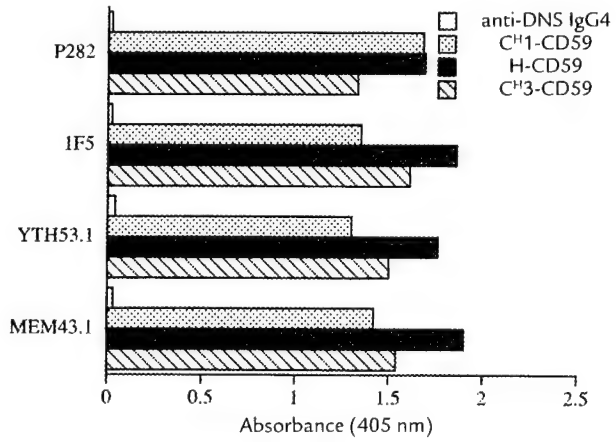

Figure 2
Binding of IgG-CD59 fusion proteins containing conformation-sensitive CD59 epitopes to dansyl. Purified IgG-CD59 fusion proteins (100 ng/ml) or anti-dansyl IgG4 control antibody were incubated in dansylated BSA–coated microtiter plates. Using standard ELISA technique, bound IgG-CD59 was determined using a panel of anti-CD59 MABs that recognize conformational epitopes on CD59. *DNS*, dansyl; *MABs*, monoclonal antibodies.

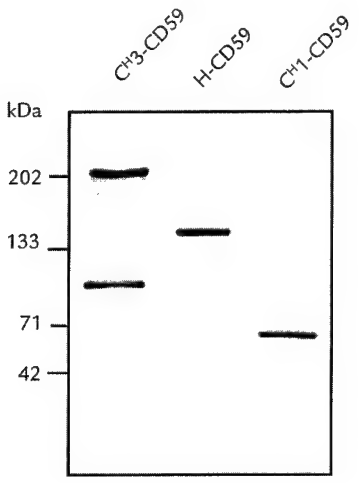

Figure 3
Anti-CD59 Western blot analysis of IgG-CD59 fusion proteins. Purified IgG-CD59 fusion proteins (20 ng) were separated on 4%–15% acrylamide nonreducing SDS polyacrylamide gels. Proteins were transferred to nitrocellulose and CD59 immunoreactive bands detected by means of anti-CD59 MAB MEM43.

ples in 1% BSA in PBS was incubated in wells for 1 h at room temperature, and bound IgG-CD59 was detected by means of anti-CD59 MABs followed by anti-mouse IgG horseradish peroxidase–conjugated antibodies and chromogenic substrate. Protein concentration of IgG-CD59 fusion proteins was determined by either ultraviolet (UV) absorbance (42) or by using a Coomassie protein assay kit (Pierce Chemical Co., Rockford, Illinois, USA).

Fusion protein purification. IgG-CD59 proteins were purified from culture supernatant by anti-CD59 affinity chromatography. Purified anti-CD59 MAB 1F5 or P282 was coupled to HiTrap NHS-activated affinity columns (Pharmacia Biotech, Piscataway, New Jersey, USA), as described by the manufacturer. Culture supernatants containing IgG-CD59 were adjusted to pH 7.5 and applied to affinity columns at a flow rate of 0.5–1 ml/min. The column was washed with 6–8 column vol of PBS, and the fusion protein was eluted with 2–3 column vol of 0.1 M glycine, pH 2.6. The fractions containing fusion protein were collected into tubes containing 1 M Tris buffer, pH 8.0, for neutralization, and dialyzed against PBS.

SDS-PAGE and Western blotting. Purified IgG-CD59 fusion proteins were separated in SDS-PAGE 4%–15% acrylamide gradient gels (Bio-Rad Life Science Research, Hercules, California, USA) under nonreducing conditions by standard procedures (48). Gels were stained with Coomassie blue. For Western blotting, separated proteins were transferred to a nitrocellulose membrane, and the membrane was probed with anti-CD59 MAB MEM43 at a 1:500 dilution in TBS buffer (Bio-Rad Life Science Research) containing 3% nonfat milk. After washing, the membrane was incubated with alkaline phosphatase–conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, Missouri, USA) at a 1:2,500 dilution in TBS/3% nonfat milk. The membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma Chemical Co.) to reveal CD59-containing bands.

BSA-dansyl labeling of CHO cells. CHO cells were labeled with dansyl by coupling dansylated BSA to the CHO cell surface. To couple dansyl to BSA, 16 mg dansyl (Sigma Chemical Co.) dissolved in 1 ml of acetone was added to 19 ml BSA (100 mg in 19 ml of Na_2CO_3, pH 9.5) dropwise at 4°C. The solution was stirred at 4°C overnight. Excess insoluble dansyl was removed by centrifugation. Unbound ligand was removed using a G25 Sephadex column. The collected G25 flowthrough was dialyzed against 0.85% NaCl, pH 7, at 4°C overnight, and BSA-dansyl concentration was determined by a Coomassie protein assay kit. To label CHO cells with dansylated BSA, 20 µl of dansylated BSA (5 mg/ml) was added dropwise to 2×10^6 cells suspended in 0.1 ml of 0.85% NaCl, and 1.5 ml of $CrCl_3$ (13.2 µg/ml in 0.85% NaCl) was then added. The cell suspension was incubated at 30°C for 30 min with gentle rotation. The cells were washed twice with PBS, and dansyl labeling of cells was confirmed by flow cytometry (excitation, 362 nm; emission, 550 nm).

Flow cytometry. To detect IgG-CD59 binding to dansyl-labeled CHO cells, cells were incubated with fusion proteins at ~2 µg/ml final concentration (30 min at 4°C). Cells were washed twice in DME M and incubated with anti-CD59 MAB MEM43 (1:500;) 30 min at 4°C). After washing, FITC-conjugated anti-mouse IgG (Sigma Chemical Co.) was added (1:200; 30 min at 4°C). Cells were then washed, fixed with 2% paraformaldehyde in PBS, and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, (San Jose, California, USA). Simultaneous determination of dansyl-labeled and viable cells in a mixed cell population was performed by two-wavelength fluorescence analysis, using a Coulter Epics Elite(Coulter corp., Miami, Florida, USA). A mixture of unlabeled and dansyl-labeled CHO cells, both antibody sensitized (see below), was incubated with 7.5% (final) NHS for 45 min at 37°C, either with or without C_H1-CD59 fusion protein. Propidium iodide (PI) (10 µg/ml final) was added to cells, and cells were analyzed for fluorescence at an excitation of 362 nm (to detect dansyl labeling) and 565 nm (to detect dead cells that have taken up PI).

Complement lysis assays. CHO cells at 60%–80% confluence were detached with versene (GIBCO BRL), washed once, and resuspended to 10^6/ml in DMEM. Cells were sensitized to complement by adding rabbit anti-CHO cell membrane antiserum (10% final concentration) to cells. An equal volume of NHS diluted in DMEM was then added. After 45 min at 37°C, cell viability was determined by either trypan blue exclusion (both live and dead cells counted) or by adding PI (10 µg/ml) and measuring the proportion of PI-stained dead cells by flow cytometry (44). Cells were lysed with 0.01% saponin for 100% lysis controls, and heat-inactivated NHS was used for background lysis. Cell lysis assays were typically performed in 1.5-ml microfuge tubes in a final volume of 100 µl. To determine the effect of IgG-CD59 fusion proteins on cell lysis, purified fusion protein (or anti-dansyl IgG4 control) in PBS was added to dansyl-labeled cells, together with anti-CHO cell sensitizing antiserum (10% final concentration), and the cells were preincubated for 15 min before the addition of different concentrations of NHS, as indicated in our figures.

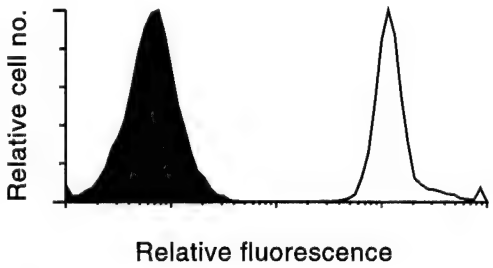

Figure 4
Dansyl labeling of CHO cells. Fluorescent BSA-dansyl conjugate was coupled to the CHO cell surface, and the cells were analyzed by flow cytometry (excitation, 340 nm; emission, 565 nm). The relative fluorescence of cells coupled with BSA alone (*shaded area*) and dansylated BSA (*unshaded area*) is shown. *CHO*, Chinese hamster ovary.

Results

Construction of antibody-CD59 fusion proteins. cDNA encoding the 77 amino acids of mature CD59 was inserted at the 3′ end of the coding sequence for various IgG heavy-chain fragments. The resulting constructs encoded a predicted set of fusion proteins consisting of CD59 joined to an antibody-combining site at the end of C_H1, after the hinge, and after C_H3 (Fig. 1). Each construct contained human IgG constant-region genes joined to a mouse anti-dansyl variable region (38). The C_H1-CD59 and H-CD59 constructs (Fig. 1) contained human IgG3 constant regions. The C_H3-CD59 fusion was constructed with a human IgG4 constant region. The IgG4 constant region was used in the C_H3 construct because IgG3 Fc, but not IgG4 Fc, activates complement.

Expression and characterization of antibody-CD59 fusion proteins. Expression vectors containing heavy chain–CD59 fusion constructs were transfected into the TWS2 cell line that produces an anti-dansyl light chain (see Methods). Transfectoma clones secreting IgG-CD59 proteins with specificity for dansyl were identified by assaying culture supernatant by ELISA. High-expressing clones were selected. IgG-CD59 fusion proteins were purified from culture supernatant by anti-CD59 affinity chromatography. Analysis of the purified fusion proteins by ELISA confirmed that each protein construct specifically recognized dansyl, and that the IgG-CD59 constructs that were bound to dansyl were recognized by a series of MABs specific for conformational epitopes on CD59 (Fig. 2). The amount of purified fusion protein isolated from culture medium was estimated at 1 μg/ml for C_H1-CD59 and 0.3 μg/ml for H-CD59 and C_H3-CD59.

SDS-PAGE and anti-CD59 Western blotting revealed that purified C_H1-CD59 and H-CD59 have molecular weights of 65,000 and 140,000, respectively (Fig. 3). These molecular weights are consistent with the predicted molecular weights of Fab-CD59 and $F(ab')_2$-$(CD59)_2$. With the C_H3-CD59 preparation, intermediate antibody chain assemblies are seen. According to molecular-weight analysis, and consistent with previous data on the secretion of recombinant IgG4 (49), C_H3-CD59 consists predominantly of heavy chain–light chain dimers (H_2L_2) and HL forms (molecular weights of 200,000 and 100,000, respectively).

H_2L_2 and HL forms would both contain dansyl-combining sites, and as predicted, BSA-dansyl–coupled agarose immunoprecipitated both major C_H3-CD59 forms (data not shown).

Targeting of IgG-CD59 fusion proteins to cell surfaces. To test targeting and complement inhibitory activity of the targeted IgG-CD59 fusion proteins, CHO cells were labeled with BSA-dansyl. Cell-surface labeling with fluorescent BSA-dansyl was demonstrated by flow cytometric analysis of cells using UV excitation (Fig. 4). Specific targeting of each IgG-CD59 fusion protein to dansyl-labeled CHO cells, but not to unlabeled cells, was shown by means of immunofluorescent flow cytometry using anti-CD59 antibodies (Fig. 5). In a separate experiment performed by anti-CD59 immunofluorescent flow cytometry, it was shown that at a similar molar input concentration of H-CD59 and C_H3-CD59, a similar relative level of CD59 was bound to the cell surface; an input concentration of 160 nM resulted in a relative mean fluorescence of 326 and 358 (corrected for fluorescence of control CHO cells) for H-CD59 and C_H3-CD59, respectively.

Protection of cells from complement-mediated lysis by IgG-CD59 fusion proteins. Antibody-sensitized CHO cells are efficiently lysed by human serum complement. However, the incubation with IgG-CD59 fusion proteins provided CHO cells with some protection from complement-mediated lysis as might be expected from the presence of soluble CD59 (Fig. 6, *top*). Nevertheless, in comparison to unlabeled CHO cells, dansyl-labeled CHO cells were much more effectively protected from lysis by the IgG-CD59 constructs (Fig. 6, *bottom*). These data show that the targeting of the IgG-CD59 constructs to the cell surface significantly enhances the ability of IgG-CD59 to protect the targeted cells from complement-mediated lysis, and indicate that for CD59 to function effectively, it must be positioned close to the site of MAC formation. This conclusion was further supported by the relative effectiveness of the different IgG-CD59 fusion proteins. At similar input concentrations, C_H1-CD59 was slightly more effective at protecting dansyl-labeled CHO cells than H-CD59, where-

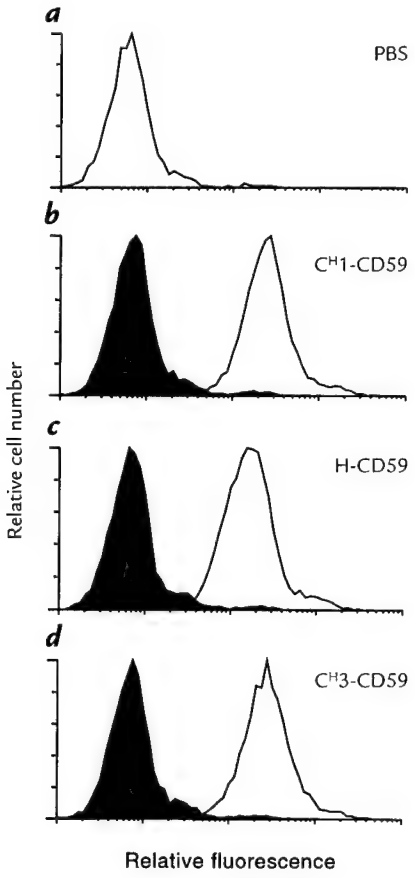

Figure 5
Specific binding of IgG-CD59 fusion proteins to dansyl-labeled cells. Unlabeled or BSA-dansyl–labeled cells were incubated with PBS (***a***) or with IgG-CD59 fusion protein (***b–d***). Binding of IgG-CD59 to cells was detected by flow cytometry using anti-CD59 MAB MEM43 and appropriate FITC-labeled secondary antibody. IgG-CD59 binding to unlabeled cells (*shaded areas*) and dansyl-labeled cells (*unshaded areas*) is shown in *b–d*. The figure shows FITC fluorescence, which was separated from dansyl fluorescence by gating.

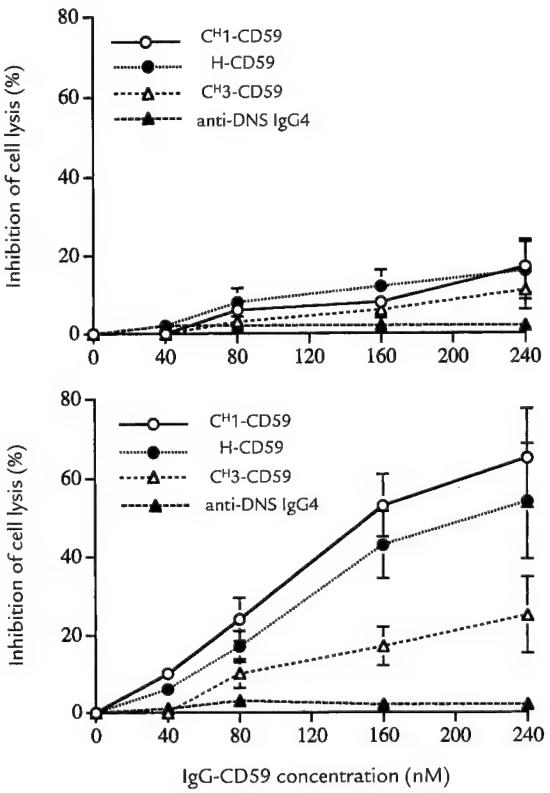

Figure 6
Inhibition of complement-mediated cell lysis by IgG-CD59 fusion proteins. CHO cells were incubated with the indicated concentration of IgG-CD59 fusion protein or IgG4 control antibody and then sensitized to complement using anti-CHO cell membrane antiserum. Human serum, to a final concentration of 10% (resulting in 75%–90% lysis of unprotected CHO cells), was then added, and cell lysis was determined after 45 min at 37°C. The figure shows the dose response of IgG-CD59 fusion proteins on complement-mediated lysis of unlabeled (*top*) and dansyl-labeled (*bottom*) CHO cells. Background lysis (cells incubated in heat-inactivated human serum) was <10% and was subtracted. Results are mean ± SD of five determinations.

as the relative effectiveness of C_H3-CD59 was less than half that of the other two constructs (Fig. 6). Thus, the farther CD59 was positioned from the antibody-combining site, the less effective an inhibitor it was. In interpreting these data with regard to the relationship between CD59 functional activity and its proximity to the membrane, it is important to note that similar molar input concentrations of H-CD59 and C_H3-CD59 resulted in similar levels of cell-bound CD59 (see above).

The protection of CHO cells from complement-mediated lysis was dose dependent for all IgG-CD59 fusions. In control experiments, CHO cells were incubated with anti-dansyl IgG4 in place of IgG-CD59 fusion proteins; anti–dansyl IgG4 had no effect on the susceptibility of either unlabeled or dansyl-labeled CHO cells to serum complement (not shown). Dansyl labeling of cells did not affect their susceptibility to complement-mediated lysis in the absence of IgG-CD59 fusion proteins (titrations of cell lysis against serum concentration were performed but are not shown).

The ability of the IgG-CD59 fusion proteins to selectively protect targeted cells in a mixed cell population was determined. Equal numbers of unlabeled and dansyl-labeled CHO cells were mixed, and the relative proportion of unlabeled and labeled cells that were lysed by complement in the presence of either phosphate-buffered saline (PBS) or C_H1-CD59 was determined. Fig. 7*a* shows that both unlabeled and dansyl-labeled CHO cells are equally susceptible to complement in the absence of IgG-CD59 inhibitor. The concentration of serum used produced ~50% cell lysis. However, in the presence of C_H1-CD59, 80% of dansyl-labeled CHO cells survived serum treatment (Fig. 7*b*, *upper quadrants*). Consistent with data shown in Fig. 6, *top* there was also a small relative increase in the survival of unlabeled CHO cells (compare Fig. 7, *a* and *b*, *lower quadrants*). These data show that C_H1-CD59 provides selective protection to targeted (*i.e.*, dansyl-labeled) CHO cells in a mixed cell population.

Discussion

Inhibition of the complement system may provide an effective strategy for therapy of autoimmune and inflammatory conditions and disease states associated with bioincompatability. A safe and effective pharmaceutical inhibitor of complement is not available, and research has largely focused on developing recombinant soluble inhibitors based on host membrane-bound complement-regulatory proteins, or on developing complement-specific antibodies (50).

In the absence of serum, native and recombinant CD59 containing a GPI anchor will spontaneously insert nonspecifically into cell membranes (51–54) and effectively protect cells from complement-mediated lysis. However, in the presence of serum, CD59 is not effective, probably due to its binding to lipoproteins (55). The effectiveness of soluble CD59 against serum complement–mediated lysis is improved by removal of its GPI anchor, but its activity relative to membrane-bound CD59 is still low (9, 51). In an attempt to develop an improved CD59-based complement inhibitor, we have examined the feasibility of targeting CD59 activity to specific tissues. We report the generation of recombinant IgG-CD59 chimeric fusion proteins that retain both serum complement inhibitory activity and antigen-binding specificity. The IgG-CD59 fusion proteins can be targeted to a specific cell surface and provide the targeted cell with protection from complement-mediated lysis. Untargeted IgG-CD59 fusion proteins were much less effective than their cell-targeted counterparts at inhibiting MAC-mediated cell lysis, indicating that the normal functioning of CD59 requires that CD59 be positioned close to the site of MAC formation. This feature of CD59 function is in contrast to that of inhibitors of complement activation (CR1, DAF, MCP), which function effectively as soluble untargeted proteins.

The univalent C_H1-CD59 fusion protein was the construct most effective at protecting targeted cells from complement-mediated lysis, even though H-CD59 and C_H3-CD59 both contain dimerized CD59 and bivalent antigen-binding sites. C_H3-CD59 effectively bound to targeted cells but did not provide efficient protection from complement-mediated lysis. It has been reported previously that some molecules fused at the end of CH3 lose activity (56, 57). However, the relative ineffectiveness of C_H3-CD59 may be related to its larger size, in

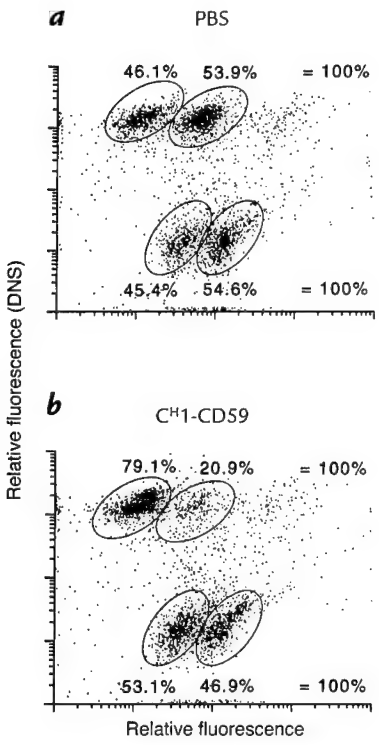

Figure 7
Selective protection of IgG-CD59 targeted cells in a mixed cell population. PBS (***a***) or C_H1-CD59 (160 nM) (***b***) was added to a mixed population of unlabeled and dansyl-labeled CHO cells. Cells were sensitized to complement, and human serum was added, to a final concentration of 7.5% (resulting in ~50% cell lysis of unprotected cells). After 45 min at 37°C, PI was added and the cells were analyzed by dual-wavelength flow cytometry. Dansyl-labeled cells are identified by ultraviolet excitation (*upper quadrants*) and dead cells are identified by uptake of PI (*right-hand side*). *PI*, propidium iodide.

whichCD59 is likely held at a greater average distance from the targeted cell membrane. Such a conclusion is consistent with our data indicating that CD59 must be in close proximity to the membrane to bind the assembling MAC and prevent cell lysis. In addition, it was shown previously that recombinant membrane-anchored CD59-DAF fusion proteins retained CD59 function only when CD59 was linked directly to the membrane and not when fused distal to DAF (58). In the current study, although CD59 is attached distal to the IgG antigen-combining site, the binding of IgG-CD59 via its antigen-binding site(s) does not necessarily fix CD59 at a perpendicular distance from the membrane, as is likely for membrane-anchored DAF-CD59 fusion proteins.

Clearly, the spatial relationship between CD59 and the site of MAC assembly is an important consideration for CD59-based therapeutic complement inhibitors. The linear distance of the spacer peptide used at the IgG COOH-terminus in the IgG-CD59 fusion proteins is about 50 nm. The average diameter of CD59 is about 25 nm, and computer models of IgG-CD59 fusion proteins revealed that a much shorter spacer would be unlikely to interfere with the protein folding and function of either fusion partner. Computer modeling also revealed that it may be possible to prepare a functional construct by linking CD59 to the NH_2-terminus of the variable region. Such a construct would place the antigen-binding site and the proposed CD59 active site (44, 59) in very close proximity to each other. In these studies we have used antibodies specific for the hapten dansyl to protect dansylated CHO cells. However, with the available vectors, it is straightforward to change the binding specificity of the antibody. Therefore, this approach potentially can be used to provide protection to any cell population recognized by a specific antibody. Potential targets include tissue-specific antigens, markers of inflammation (such as cell adhesion molecules), and foreign antigens on xenotransplanted tissues and organs. However, the location of the epitope on a target antigen will affect the position of bound CD59 relative to the cell membrane and is likely to be an important consideration in the design of an effective tissue-specific IgG-CD59 protein.

Many proteins have been fused with Fc regions for the purpose of increasing circulatory half-life and increasing their binding affinity due to dimerization by antibody chains. Inhibitors of complement activation (*e.g.*, CR1 [60] and mouse Crry [61]) have previously been fused to IgG fragments but have not been targeted to cells. It is possible that inhibitors of complement activation that act at an early step in the amplification cascade may be more protective of complement-mediated injury than CD59 (an inhibitor of the terminal pathway). However, because early complement pathway activation products are important in host response to infection and immune complex catabolism, there may be circumstances when inhibiting C5b-9 formation, but leaving the complement activation pathway intact, may be of benefit. In this respect, the terminal C5b-9 complex has been implicated in the pathogenesis of several diseases. Our data indicate that only CD59 that is targeted and bound to the site of MAC formation is likely to be a clinically effective inhibitor. The reported approach of targeting complement inhibition may also be appropriate for inhibitors of complement activation, because their targeting would permit a much lower effective serum concentration and would minimize undesirable systemic effects.

Acknowledgments

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Surface Antigen Expression and Complement Susceptibility of Differentiated Neuroblastoma Clones

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Abstract

Human neuroblastoma cell lines typically consist of heterogenous subpopulations of cells that are morphologically and biochemically distinct. The cell types are characterized as neuroblastic (N-type), substrate-adherent Schwann-like (S type), or intermediate (I). These cell types can undergo spontaneous or induced transdifferentiaition in vitro. We investigated the complement sensitivity of different neuroblastoma cell lines and of matched sets of cloned N and S-type neuroblastoma cell lines. Human neuroblastoma cell lines that consisted predominantly of a neuroblastic phenotype were shown to be significantly more susceptible to human complement-mediated lysis than cell lines of other cancer types. Complement sensitivity of neuroblastoma cell lines was correlated with low levels of CD59, DAF and MCP expression. We found that cloned S-type neuroblastoma cells were much more resistant to complement-mediated lysis than cloned N-type cells. The increased complement resistance of S-type cells was shown to be due to increased expression of membrane-bound complement inhibitors. CD59 was the single most important protein in providing S-type cells with protection from complement lysis. S-type cells were also found to express lower levels of GD2, a target antigen for a complement activating monoclonal antibody currently in clinical trials for neuroblastoma immunotherapy. The ability of S-type cells to evade complement, and the ability of S-type cells to differentiate into the more tumorigenic N-type cells, may represent a mechanism of tumor survival and regrowth, a phenomenon often observed with this cancer.

Introduction

Neuroblastoma is one of the most common extracranial solid tumors of children and is often lethal in patients who present with metastatic disease. Although these tumors may respond well to chemotherapy, and spontaneous tumor regression is sometimes observed, it has a propensity to recur, sometimes after long periods of quiescence, eventually killing the patient. The molecular mechanisms underlying disease progression and tumor regrowth are not well understood [1].

Neuroblastomas exhibit diverse morphologies with tumors composed generally of a mixture of neuroblasts, ganglion cells, Schwann-like and stromal cells. Since the initial description of distinct N and S cells by Biedler et al, subclones have been derived from established neuroblastoma cell lines [2]. Cells with intermediate morphology (I-type) have has also been cloned, and the three subtypes can interconvert or transdifferentiate either spontaneously or following chemical induction. In vivo correlates of these various clonal subtypes have not been definitively determined, but it is generally believed that S-type cells exist and may be masquerading as stromal or Schwann-like cells. Although some stromal cells in human neuroblastoma may derive from normal tissues, the presence of S-type cells in human neuroblastoma is a real possibility.

It is not clear how transdifferentiation between the different morphologic phenotypes might modify tumor behavior and response to treatment. It has been hypothesized that S-type cells represent a more differentiated benign cell type and that tumor regression, either spontaneous or as a result of therapy, may parallel transdifferentiation from N to S cells [2]. It is also possible that S-type cells, and their ability to differentiate to more tumorigenic N cells, represent an important link between tumor regression and frequently observed tumor recurrence. There have been many studies on N and S cell differentiation and on the molecular basis for N cell tumorigenicity. However, these studies have not addressed the relative resistance of the cell types to anti-tumor reagents and to host defense mechanisms.

Complement resistance is likely to play an important role in tumor cell survival, and may contribute to tumor cell escape from immune surveillance and present obstacles to effective antibody-mediated immunotherapy. The Complement effector systems involved in the immune response to tumor cells include amplification of inflammatory response, recruitment and activation of immune effector cells and direct complement-mediated cytolysis. Complement activation is controlled on the surface of host cells by the membrane-bound proteins decay-accelerating factor (DAF), membrane cofactor protein (MCP) and complement receptor 1 (CR1). These proteins inhibit formation of the C3 convertase, an enzymatic complex that amplifies the complement cascade. The terminal complement pathway is inhibited by membrane-bound CD59 which binds to the assembling membrane attack complex (MAC or C5b-9) and prevents cytolysis. CD59 together with DAF and/or MCP are expressed by almost all primary tumors and tumor cell lines that have been examined, and are often upregulated on tumor cells. In this study we investigate the expression of complement inhibitors by various neuroblastoma cell types, and the susceptibility of these cells to complement-mediated lysis.

Materials and Methods

Cell lines

SK-N-ER is a neuroblastoma cell line established established at Memorial Sloan-Kettering Cancer Center. LAN-1 neuroblastoma cell line was obtained from Dr. Robert Seeger of UCLA, Los Angeles, CA. Seven clones of the neuroblastoma cell line LAN-1 were derived as previously described [3], and the derived N-type and S-type cloned cell populations (55N, 5S, 66N, 6S) were kindly provided by Dr. Robert Ross, Fordham University, New York, NY. NMB-7 (neuroblastoma) was provided by Dr. Liao of McMaster University, Ontario, Canada. The melanoma cell line HTB-63 was provided by Dr. A. N. Houghton (Memorial Sloan-Kettering

Cancer Center, New York). The ovarian cell line SKOV3 was provided by Dr. M. L. Disis (University of Washington, WA). The breast cancer cell line BT474 was purchased from the American Type Culture Collection. HTB-63 and SKVO3 were maintained in McCoy's S5A medium (GIBCO BRL, Grand Island, NY) containing 10% fetal calf serum. All other cell lines were passaged in RPMI 1640 media supplemented with 10% heat-inactivated defined bovine calf serum (Hyclone, Logan, UT), 2mM glutamine. All media contained 100 U/ml of penicillin and 100 ug/ml of streptomycin and incubation was at 37°C in 5% CO_2.

Antibodies and complement

Rabbit antisera to tumor cell membranes that was used to sensitize the various tumor cell lines to complement was prepared by standard techniques [4]. Cell membranes of each cell line were prepared by Dounce homogenization of cells in hypotonic media (10 mM sodium phosphate, pH 8) and subcellular fractionation to remove nuclei and mitochondria. Anti-GD2 3F8 monoclonal antibody [5] and the tumor-selective 8H9 monoclonal antibody [6] were described previously. Anti-human CD59 monoclonal antibody YTH53.1 [7] was a gift from Dr. B. P. Morgan (University of Wales, Cardiff, UK), anti-DAF polyclonal antibody and monoclonal antibody 1H4 [8] were gifts from Dr. T. Kinoshita (Osaka Univeristy, Japan) and anti-MCP monoclonal antibody M75 [9] was a gift of Dr. D. M. Lublin (Washington Univeristy, St. Louis, MO). Anti-DAF monoclonal antibody 1A10 was described previously [8]. $F(ab)_2$ antibody fragments of anti-CD59 YTH53.1 and anti-DAF 1H4 were prepared by pepsin digestion using an $F(ab)_2$ preparation kit from Pierce (Rockford, IL) according to supplied instructions. FITC-conjugated antibodies used for flow cytometry were purchased from Sigma (St. Louis, MO). Normal human serum (NHS) was obtained from the blood of healthy volunteers in the laboratory and stored in aliquots at -70 °C until use.

flow cytometry and Western blot analyses

Analysis of cell surface protein expression was performed by flow cytometry using appropriate antibodies as previously described [10]. Isotype matched control antibodies were used in experiments. Anti-DAF Western blotting was performed on cell membrane preparations that were prepared as described above. SDS-PAGE and immunoblotting was performed as described in a previous study that analyzed DAF expression on neuroblastoma cell lines [11]. The anti-DAF monoclonal antibody 1H4 was used for Western blot analysis. Membrane preparations from the equivalent of approximately 3 x 10^5 cells were loaded per lane for the neuroblastoma cell lines and from the equivalent of approximately 1 x 10^5 cells for SKOV3; the Western blot data shown is qualitative and was performed to confirm data on DAF expression obtained from flow cytometry.

complement lysis assays

Complement-mediated cell lysis was determined by a standard ^{51}Cr release assay [12]. Briefly, cells at 50-80% confluency were detached with versene/EDTA (Gibco), washed once and resuspended in EMEM/10% heat inactivated fetal calf serum. Cells were preloaded with ^{51}Cr at a concentration of 1 x 10^7/ml (2h/37 ^oC), washed in complete media and resuspended to 1 x 10^6/ml. Rabbit anti-cell membrane antisera at a final concentration of 10% diluted in EMEM/10% fetal calf serum, or monoclonal antibody 3F8 at 15 ug/ml was added and the cells incubated on ice for 30 min. Cells were centrifuged and resuspended to 1 x 10^6/ml in EMEM/10% fetal calf serum. Equal volumes of cells and serum dilutions were incubated for 60 min. at 37 ^oC, and cell lysis determined by measuring released radioactivity. In some experiments, lysis was determined by trypan blue exclusion [13] (with similar results). Complement lysis assays of neuroblastoma cell lines were also

performed using monoclonal antibody 8H9 together with anti-IgG1 polyclonal antibody to sensitize cells to complement. Monoclonal antibody 8H9 is IgG1 and recognizes a tumor-selective surface antigen on neuroblastoma cells [6]. Cells were incubated first with 8H9 at 10 ug/ml for 30 min/4°C, and purified rabbit anti-mouse IgG1 polyclonal antibody at 15 ug/ml (Sigma) was then added. A secondary anti-IgG1 complement activating antibody was necessary because mouse IgG1 does not activate complement. At similar antibody concentrations, similar levels of anti-IgG1 bound to both 5S and 55N cells as determined by flow cytometry (see results).

The effect of anti-complement inhibitor blocking antibodies and $F(ab)_2$ fragments on complement-mediated lysis was performed essentially as described [14-16]. The function blocking activity of anti-CD59 YTH53.1 [14,17], anti DAF 1H4 [15] and anti-MCP M75 [16] have been previously characterized. Cells were preincubated with 50 ug/ml blocking antibody or $F(ab)_2$ fragment for 30 min. before the addition of sensitizing antibody, and lysis was then determined. Complement inhibitor blocking experiments were performed with whole antibodies, and for YTH53.1 and 1H4, with $F(ab)_2$ fragments. The results were essentially similar whether whole antibody or $F(ab)_2$ fragments were used.

Results

Sensitivity of neuroblastoma and other cancer cell lines to lysis by complement

Three neuroblastoma cell lines and a representative cell line from three other types of cancer were assayed for their sensitivity to human serum. All of the neuroblastoma cell lines tested were effectively lysed by human complement (fig. 1). In contrast, the other cancer cell lines were relatively complement resistant, even at high concentrations of human serum. This finding is generally consistent with studies using various other cancer cell lines. Rabbit antisera raised against

membrane preparations from each cell line was used to sensitize the tumor cells to complement, and at the antiserum concentration concentration used in complement lysis assays, all cell lines stained with a similar saturating mean fluorescence when analyzed by flow cytometry. However, it is possible that differences in the sensitizing antibodies may account for the difference in the observed lysis. The antisera may also contain antibodies against membrane complement inhibitors that may bias the results, although we could not detect purified CD59 on a Western blot using the antisera (not shown). For this reason, we compared the complement susceptibilty of HTB-63 and NMB-7 using the anti-GD2 monoclonal antibody, 3F8, as sensitizing antibody. These two cell lines were found to exhibit a similar mean fluorescence when stained by means of 3F8 (HTB-63 = 486, NMB-7 = 437), but the melanoma cell line was considerably more resistant to lysis by human complement when sensitized with 3F8 (fig. 2), consistent with data obtained using polyclonoal antisera. Also, The relative sensitivities of the cell lines to complement was the same when various other complement activating anti-tumor antigen monoclonal antibodies were used to sensitize tumor cells (anti-HER2 for BT474 and SKOV3 cell lines that both express high levels of the HER2/erbB2 antigen, anti-GD3 for HTB-63 and anti-GD2 for neuroblastoma cell lines), although antigen density and relative binding of the different antibodies was not quantitated (data not shown).

Expression of complement inhibitors

Each cell line was assayed for relative expression of membrane-bound complement inhibitors by flow cytometry. Data in fig. 3 show that the relative expression of CD59, MCP and DAF on neuroblastoma cell lines were all low compared to expression of these complement inhibitory proteins on the other cancer cell types. Thus, the complement resistance of neuroblastoma and the other cancer cell lines correlated with the relative overall expression levels of complement inhibitors. Of note, we detected DAF expression on the surface of the neuroblastoma cell lines, whereas previous studies have failed to detect expression of DAF on various neuroblastoma cell lines [11,18].

To ensure that the 1H4 anti-DAF monoclonal antibody that we used for flow cytometry was not cross reacting with a neuroblastoma cell surface antigen, we performed flow cytometric analysis using different anti-DAF antibodies (1A10 monoclonal antibody and anti-DAF polyclonal); the same relative levels of DAF expression were found (not shown). To further confirm our data, we also performed anti-DAF Western blot analysis of neuroblastoma cell membranes. Fig 4 shows the presence of DAF of the expected molecular weight (60 kDa) in LAN-1 and SK-N-ER neuroblastoma cell membranes. We did not detect DAF in NMB-7 membrane preparations by this method, but the level of DAF expression by this cell line as indicated from flow cytometry was extremely low. The second band of lower molecular weight reacting with anti-DAF antibody observed in the LAN-1 membrane may be a degredation product.

Analysis of N and S cell types

As noted, neuroblastoma consists of diverse morphologies. We next analyzed cloned matched sets of N and S type cells, derived from the LAN-1 cell line, for their susceptibility to complement-mediated lysis. We wished to use the clinically relevant antibody 3F8 to sensitize the N and S type cells to complement. 3F8 is a complement activating antibody currently in clinical trials that recognizes GD2, an antigen overexpressed on neuroblastoma. However, S cells express significantly lower levels of GD2 relative to N type cells and the parental LAN-1 cell line (fig. 5) (see also [19]). This finding means that 3F8 is not a suitable complement activating antibody for comparing the complement sensitivity of N and S cells, but also has possible implications regarding the survival of S type cells in vivo following 3F8 immunotherapy. A different tumor-selective antibody, 8H9 [6], was found to stain LAN-1 and the matched set of 5S and 55N cells with a similar mean fluorescence when analyzed by flow cytometry (fig. 5). 8H9 recognizes an undefined tumor-selective antigen and is a candidate for antibody-targeted therapies [6]. 8H9 was therefore used to target complement to the cell surface in complement lysis assays of 5S, 55N and LAN-1. Because

8H9 is a non complement activating mouse IgG1 isotype, we used 8H9 together with polyclonal anti-IgG1 antibody to sensitize the cells to complement (see methods). Fig 6 shows that 55N and the parental cell line LAN-1 were senstive to lysis by human complement, with 55N being slightly more resistant. The 5S cells however, were almost completely resistant to lysis by complement. The fact that the parental LAN-1 cell line is the most senstive to complement-mediated lysis likely just represents the average of a heterogenous population containing highly sensitive clones. Cell lines in passage become heterogenogenous over time and the LAN-1 cell line has been in passage over several years, whereas the N and S cell variants were cloned out with a limited number of passages.

We next analyzed 5S and 55N for expression of complement inhibitors. Fig. 7 shows that the intensity of staining for CD59, DAF and MCP is considerably higher (between 2 to 4 fold) for complement resistant 5S compared to complement sensitive 55N. Similar relative staining intensities were found for another cloned set of N and S type cells, 6S and 66N (fig. 7). Thus, the complement resistance of 5S compared to 55N correlated with a higher relative level of complement inhibitor expression on 5S. To firmly establish that the resistance of 5S cells to complement was due to the increased expresison of complement inhibitors, the complement susceptibility of the 5S cells was determined in the presence of antibody or $F(ab)_2$ antibody fragments that block the function of the different complement inhibitors. The data presented in fig. 8a show that blocking CD59 function on 5S enhanced complement-mediated lysis to a level comparable to that seen with 55N. Blocking DAF function had a more modest effect on enhancing complement lysis, whereas blocking MCP function had no effect. The function blocking activity of each of the monoclonal antibodies and $F(ab)_2$ fragments has been previously characterized (refer to methods). Blocking the function of complement inhibitors on 55N also enhanced complement-mediated lysis (fig. 8b). Blocking CD59 function on 55N cells had a particularly significant effect. As with 5S, blocking DAF function on 55N resulted in a more modest enhancement of complement lysis, and blocking MCP function had no effect. $F(ab)_2$ fragments of the anti-CD59 and anti-DAF antibodies were used to prevent any contribution to cell lysis from complement activation by the whole antibodies.

$F(ab)_2$ fragments of the anti-MCP antibody were not tested since the whole antibody did not enhance lysis of antibody-sensitized neuroblastoma cells.

Discussion

Neuroblastoma tumors are morphologically diverse. Neuroblastic cells predominate, but nonneuronal Schwann-like cells have been observed [20,21]. When human neuroblastoma cell lines are established in culture they spontaneously give rise to heterogenous populations of neuroblastic (N-type) and Schwann-like (S-type) cells that have distinct biochemical and morphological characteristics [2,22,23]. N type cells predominate in established neuroblastoma cell lines. The N and S type cells observed in vitro may have in vivo correlates.

S cells have limited growth potential in vivo and in vitro [23,24]. It has been suggested that S cells represent a more differentiated state, and that N to S differentiation parallels in vivo differentiation and tumor regression [2]. However, the survival of S-type cells in vivo and their ability to differentiate back into tumorigenic N-type cells may represent a mechanism of tumor cell survival and regrowth. In this context, complement evasion may be an important mechanism of survival from immune surveillance effector mechanisms or from antibody-mediated immunotherapy. We compared a matched set of N-type and S-type cell clones (5S and 55N) for their resistance to human complement, and found that S cells were much more resistant to complement-mediated lysis. Compared to the N cell clone, the S cells were found to express significantly increased levels of all three major membrane-bound inhibitors of complement. Similar relative levels of complement inhibitor expression was found on a second cloned set of S and N cells (6S and 66N). Additional data established that the increased expression level of complement inhibitors on the 5S cells was responsible for their increased resistance to complement lysis.

Results from experiments in which the function of each complement inhibitor was individually blocked indicated that CD59 was the most effective single molecule at providing protection from complement-mediated lysis. Of the two inhibitors of complement activation (DAF and MCP), only DAF neutralization enhanced complement-mediated lysis of S cells, albeit less than CD59 neutralization. Nevertheless, CD59, unlike DAF and MCP, does not directly effect complement activation and the generation of C3 and C5 activation products. It should be noted that these complement activation products, either deposited on the cell surface (C3 fragments) or released as soluble inflammatory mediators (C3a and C5a), may be important for promoting or enhancing cell-mediated cytotoxic mechanisms in vivo.

The complement susceptibility of neuroblastoma may be a significant factor in the outcome of neuroblastoma immunotherapy using unmodified monoclonal antibodies, and in this respect, the anti-GD2 monoclonal antibody 3F8 has proven relatively successful in clinical trials [25,26]. The role of S type cells in neuroblastoma is not clear, but the low level of GD2 expression and the high levels of complement inhibitor expression on S type cells may provide a mechanism for their survival from anti-GD2 and complement-mediated immunotherapy. Of further interest, data show that S cells are also more resistant to the cytostatic and cytotoxic effects of radiation and anthracyclines (N.K.V.Cheung, unpublished data). GD2 and complement inhibitor expression levels on S cells may also have implications for diagnostic procedures and bone marrow purging.

The complete elimination of S cell types may be important for long term patient survival, and tumor regrowth may be related to the ability of S type cells to survive and subsequently transdifferentiate into the more tumorigenic N type. Differential antigen expression by S type cells and their increased complement resistance may provide the basis for the ability of neuroblastoma to survive as microscopic residual disease.

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Figure legends

Figure 1. Lysis of tumor cell lines by human complement. Cells were sensitized to complement by preincubation in 10% anti-membrane rabbit antiserum. Sensitized cells were washed, exposed to the indicated concentration of human serum (37 °C/60 min), and cell lysis determined. The omission of either sensitizing antibody or the use of heat inactivated human serum in cell lysis assays resulted in a background lysis of less than 10% of test value. Figure shows representative data from 3 separate experiments.

Figure 2. Lysis of anti-GD2 sensitized HTB-63 and NMB-7 by human complement. Cells were sensitized to complement by preincubation in anti-GD2 3F8 monoclonal antibody at 15 ug/ml. Sensitized cells were washed, exposed to the indicated concentration of human serum (37 °C/60 min), and cell lysis determined. Figure shows representative data from 3 separate experiments.

Figure 3. Surface expression of complement inhibitory proteins on tumor cell lines. Cells were stained by immunofluorescence using monoclonal antibodies to human CD59 (YTH53.1), MCP (M75) and DAF (1H4) as primary antibodies. Figure shows relative mean fluorescence by flow cytometric analysis. Isotype matched antibodies of irrelevant specificity were used for controls.

Figure 4. Western blot analysis of DAF expression by tumor cell lines. Membrane preparations from the indicated neuroblastoma cell lines and the SKOV3 ovarian cancer cell line were analyzed by Western blot using anti-DAF monoclonal antibody 1H4.

Figure 5. Surface expression of tumor-associated antigens on neuroblastoma tumor cell lines. Cells were stained by immunofluorescence using monoclonal antibodies to GD2 (monoclonal antibody 3F8) and an undefined tumor-selective antigen (monoclonal antibody 8H9) as primary

antibodies. Figure shows relative mean fluorescence by flow cytometric analysis. Isotype matched antibodies of irrelevant specificity were used for controls.

Figure 6. Lysis of neuroblastoma tumor cell lines by human complement. Cells were sensitized to complement by preincubation with 8H9 and anti-IgG1 antibodies. Sensitized cells were then exposed to the indicated concentration of human serum (37 ^{o}C/60 min), and cell lysis determined. The omission of either sensitizing antibodies or the use of heat inactivated human serum in cell lysis assays resulted in a background lysis of less than 10% of test value. Figure shows representative data from 3 separate experiments.

Figure 7. Surface expression of complement inhibitory proteins on neuroblastoma tumor cell lines. Cells were stained by immunofluorescence using monoclonal antibodies to human CD59 (YTH53.1), MCP (M75) and DAF (1H4) as primary antibodies. Figure shows relative mean fluorescence by flow cytometric analysis.

Figure 8. Effect of blocking the function of complement inhibitory proteins on complement-mediated lysis of 5S and 55N cells. 5S cells (a) or 55N cells (b) were preincubated with $F(ab)_2$ fragments of anti-CD59, anti-DAF or whole IgG anti-MCP monoclonal antibody at 50 ug/ml. Cells were then sensitized to complement, exposed to the indicated concentration of human serum (37 ^{o}C/60 min), and cell lysis determined. Increasing the concentration of function blocking anti-complement inhibitor $F(ab)_2$ fragments or antibody did not further enhance complement-mediated lysis. Figure shows representative data from 3 experiments.

fig. 1

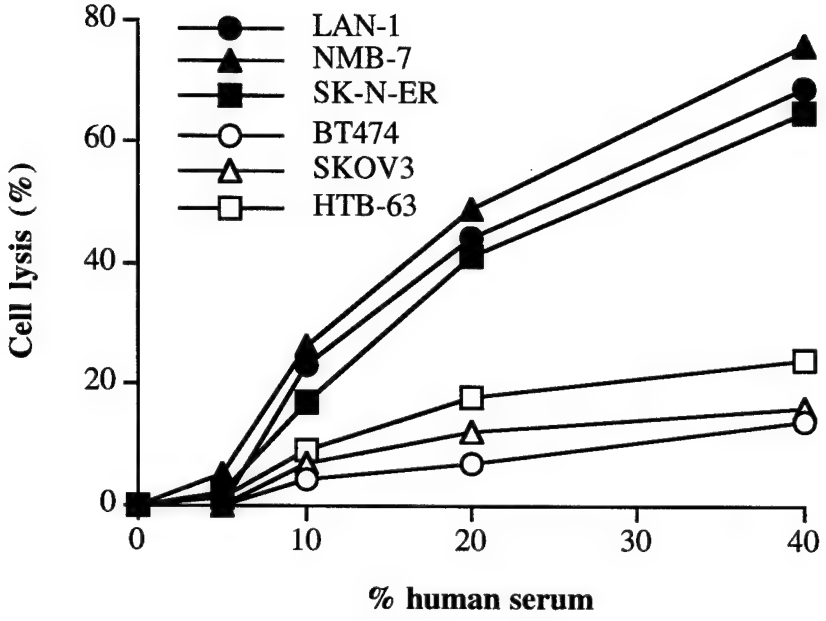

80
60
40
20
0
Cell lysis (%)
LAN-1
NMB-7
SK-N-ER
BT474
SKOV3
HTB-63
0
10
20
30
40
% human serum

Fig. 2

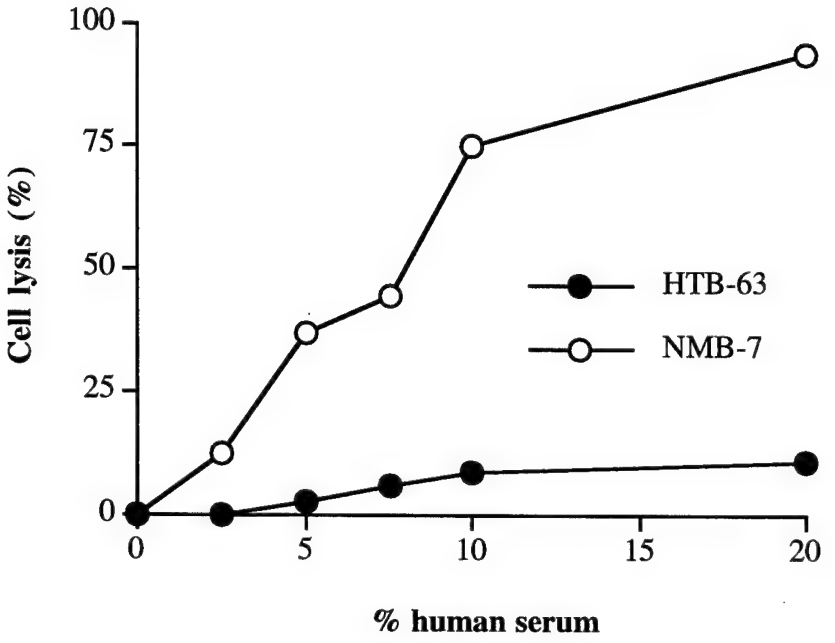


fig. 3

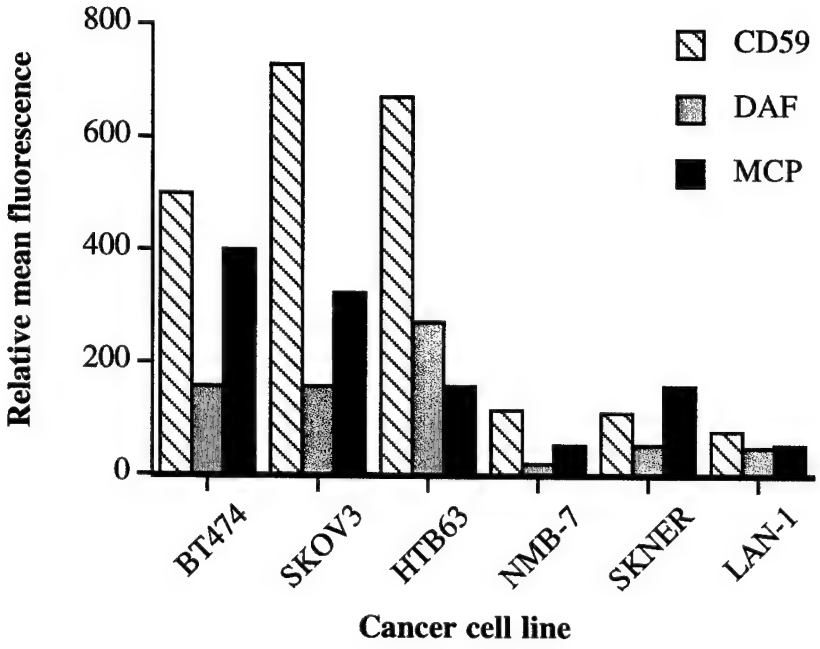

800
600
400
200
0
Relative mean fluorescence
CD59
DAF
MCP
BT474
SKOV3
HTB63
NMB-7
SKNER
LAN-1
Cancer cell line

fig. 5

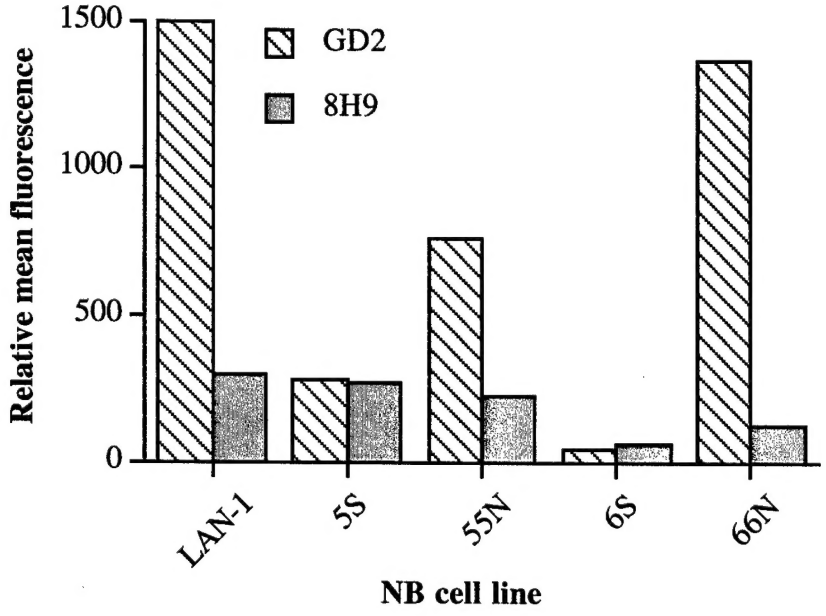

1500
1000
500
0
Relative mean fluorescence
GD2
8H9
LAN-1
5S
55N
6S
66N
NB cell line

fig. 6

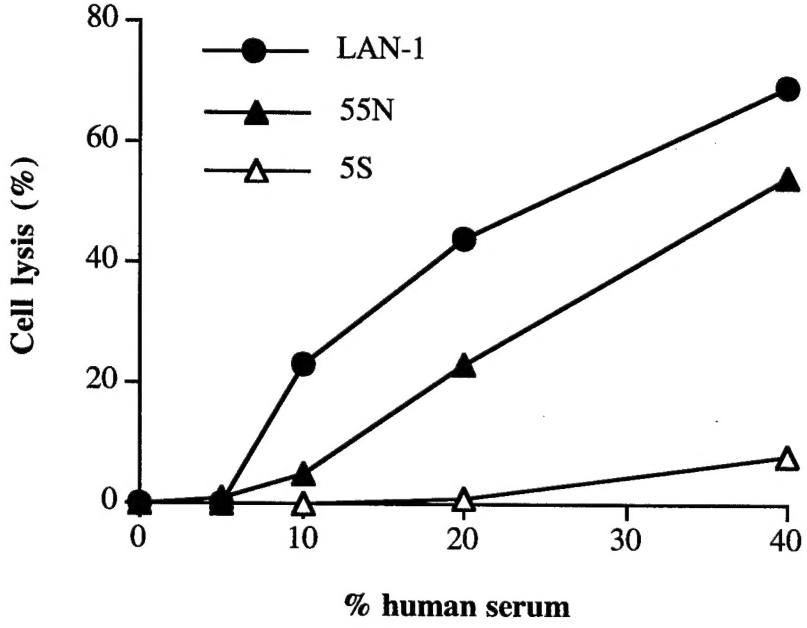


fig. 7

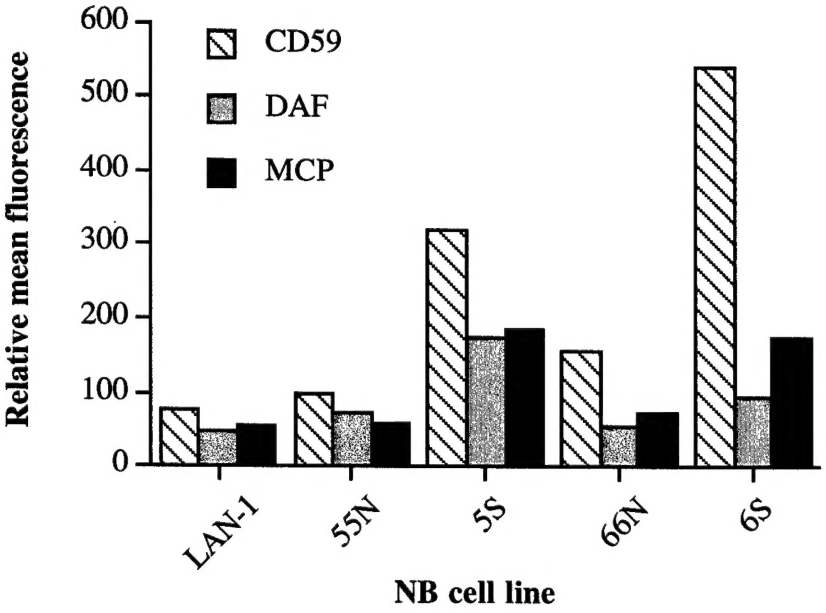
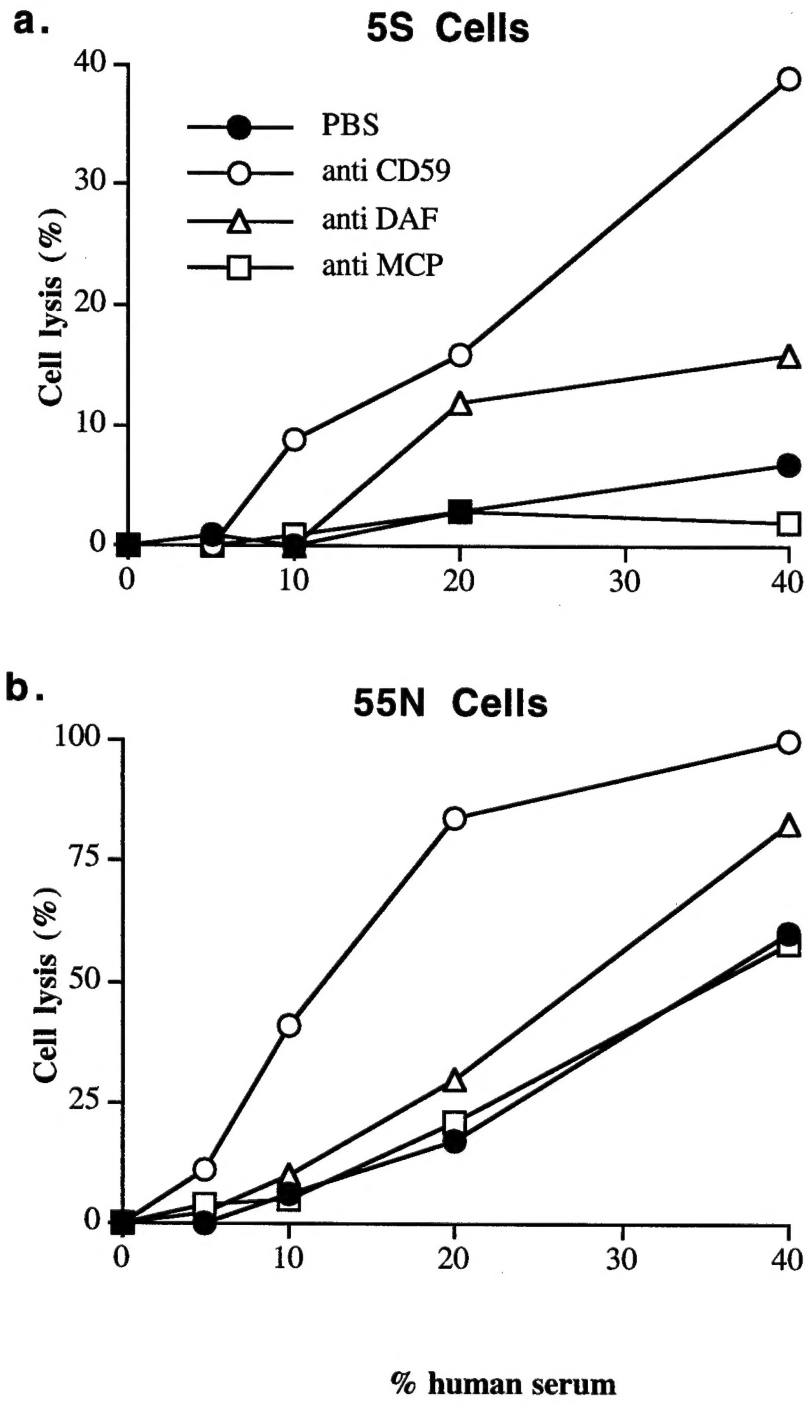

600
500
400
300
200
100
0
CD59
DAF
MCP
Relative mean fluorescence
LAN-1
55N
5S
66N
6S
NB cell line

Fig. 8


a.
5S Cells
PBS
anti CD59
anti DAF
anti MCP
Cell lysis (%)
0
10
20
30
40
b.
55N Cells
Cell lysis (%)
0
25
50
75
100
0
10
20
30
40
% human serum